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ORIGINAL ARTICLE

Interactions of α_1–proteinase inhibitor with small ligands of therapeutic potential: binding with retinoic acid

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Abstract Human α_1–proteinase inhibitor (α_1–PI), also known as α_1-antitrypsin, is the most abundant plasma serine protease inhibitor (serpin). It is best recognized for inhibition of neutrophil elastase. The α_1–PI interactions with non-protease ligands were investigated mainly in regards to those molecules that may block the aggregation of α_1–PI Z mutant. The objective of this study was to evaluate the potential of α_1–PI to bind small non-peptide ligands of pharmaceutical interest that may attain additional properties to currently available α_1–PI therapeutic preparations. Among putative ligands of bio-medical interest examined in this study, all-*trans* retinoic acid (RA) was selected due to its recently proposed roles in the lungs, and as an efficient optical probe. The results of this study, including absorption spectroscopy data, fluorescence quenching and the protein-induced chirality of the visible circular dichroism strongly suggest that α_1–PI does bind RA in vitro to non-covalent complexes of up to two moles of RA per one mole of the protein. To our knowledge, this is the first report that provides experimental evidence of the α_1–PI potential towards bi-functional drugs via a combination with RA, or potentially other molecules of pharmaceutical interest, that ultimately, may enhance currently available α_1–PI therapies.

Keywords Alpha-1 proteinase inhibitor · Antitrypsin · Retinoic acid

Introduction

Human α_1–PI is one of the best characterized serpins (see reviews by Lomas 2005; Huntington 2006; Whisstock and Bottomley 2006). α_1–PI is a multifunctional proteinase inhibitor with broad spectrum of activities (Janciauskiene et al. 2007; Petrache et al. 2006; Zhang et al. 2007; Congote 2007). Its major and the best known physiological function is to inhibit elastase in the lungs, thereby protecting the lung alveoli from being destroyed by the protease (Crystal 1996; Huntington et al. 2000; Devlin and Bottomley 2005).

Human α_1–PI is a single-chain glycoprotein containing 394 amino acid residues. It has a typical serpin structure featuring three β-sheets, nine α-helices and the reactive center loop (RCL) exposed for interaction with protease (PDB: 1hp7).

α_1–PI is the most abundant serpin in the circulation. Its deficiency in the blood and the "conformational diseases" associated with the polymerization and accumulation of the pathogenic α_1–PI Z mutant within hepatocytes has been a subject of fundamental multi-disciplinary investigation (Devlin et al. 2002; Lomas 2005; Lawless et al. 2008; Gooptu and Lomas 2008; Gooptu and Lomas 2009; Knaupp and Bottomley 2009).

Since the 1980s, the plasma-derived α_1–PI and its recombinant versions have been under continuous development for therapeutic needs, including α_1–PI products for replacement therapy to slow down a progression of emphysema in patients with inherited α_1–PI deficiency and α_1–PI topical preparations for use in dermatology (Brown

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2006; Karnaukhova et al. 2006; Mazereeuw-Hautier et al. 2006).

As the archetypal serpin, α_1–PI has a high homology and shares a conservative tertiary structure with other serpins. It is known that non-inhibitory serpins may function as binders and transporters of small hormone molecules. Some inhibitory serpins, e.g., antithrombin and protein C inhibitor can bind small hydrophobic ligands of non-proteinase nature, in addition to their inhibitory function (Jerabek et al. 2001; Huntington et al. 2003; Patston et al. 2004; Silverman et al. 2001). The interactions of α_1–PI with non-protease ligands were investigated mainly in regards to the peptides that may prevent the aggregation of Z mutant (Mahadeva et al. 2002; Zhou et al. 2004; Chang et al. 2006; Chang et al. 2008), and the osmolytes that may alter the protein conformation (Chow et al. 2001; Devlin et al. 2001; Pearce et al. 2008). Other compounds that were investigated earlier in vitro for binding with α_1–PI include cholesterol and bile acids (Janciauskiene and Eriksson 1993, 1994). Recently, a lateral hydrophobic cavity identified in α_1–PI structure (Elliott et al. 2000; Lee et al. 2001; Parfrey et al. 2003) was examined for binding small ligands which may block the aggregation of Z mutant (Mallya et al. 2007).

The objective of this study was to evaluate the potential of α_1–PI to bind small ligands of pharmaceutical interest that may attain additional properties to α_1–PI preparations and may ultimately be utilized towards development of bi-functional drugs for α_1–PI-based therapies, i.e., treatment of human emphysema and/or atopic dermatitis. The hypothesis that the efficiency of complex bi-functional treatment can be better than that of α_1–PI or ligand drug alone is a novel approach. Noteworthy, for water-insoluble drugs α_1–PI may serve as a carrier.

Among putative ligands of bio-medical interest with the indications suitable for a combination with α_1–PI, all-*trans* retinoic acid (RA) is the most prominent candidate for α_1–PI due to its multiple physiological activities (Guidas et al. 1994; Lane and Bailey 2005). RA is a small (300.4 Da) lipophylic molecule that is composed of β-ionone cycle and a conjugated polyene chain of the entire length about 15 Å (Fig. 1a). Pharmacological activities of RA are utilized in the treatment of acute promyelocytic leukemia (Huang et al. 1988; Conley et al. 1997), in chemopreventive therapy and in a variety of dermatologic uses (Treat et al. 1996; Park et al. 2000). Recently proposed role of RA in alveolar regeneration (Massaro and Massaro 1996, 1997) led to an intensive investigation of RA for treatment of emphysema (Belloni et al. 2000; Mao et al. 2002; Hind and Maden 2004; Maden and Hind 2004; Cho et al. 2005).

As both RA and α_1–PI are considered for the same indications, it is feasible that α_1–PI-based therapies may benefit from combination with RA, e.g., whereas α_1–PI does not cure, but may slow down a progression of human emphysema, the RA has been proposed as the first powerful medication that may reverse emphysematous lung damage (Massaro and Massaro 1996, 1997). The clinical benefits of each treatment still remain to be clearly proven (see, e.g., Roth et al. 2006). Nevertheless, the α_1–PI complexes with small drugs may lead to an enhanced efficacy in regards to currently available treatment. Also, binding of RA to protein may solve the issues of RA low aqueous solubility, instability and toxicity (Noy 1992, 1999). This study provides biochemical characterization of the interactions between RA and α_1–PI.

UV–vis absorption data, fluorescence quenching and the induced visible CD presented herein demonstrate that human α_1–PI inclusively binds RA in vitro to form non-covalent complexes. To the best of our knowledge, this is the first report on binding of retinoic acid to α_1–PI. This work demonstrates the feasibility for further research in this direction and supports the drug design based on α_1–PI interactions with other putative ligands of pharmaceutical interest that may attain additional properties to currently available α_1–PI therapies.

Materials and methods

Materials

All-*trans* retinoic acid, bovine pancreatic trypsin (BPT), porcine pancreatic elastase (PPE) and *p*-nitrophenyl-*p*′-guanidinobenzoate (NPGB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *trans*-Cinnamic acid and myristic acid (Acros Organic) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Human α_1–PI preparation received as a gift for research purposes from Aventis Behring (ZLB) was used throughout the study. Reconstituted human α_1–PI from Athens Research and Technology (AR) (Athens, GA, USA) was utilized for comparative evaluation. Ethyl alcohol (EtOH) anhydrous, USP grade was purchased from Warner-Graham (Cockeysville, MD, USA). Phosphate buffered saline (PBS) pH 7.4 was from Quality Biological, Inc. (Gaithersburg, MD, USA). Other chemicals were analytical grade from Fisher Scientific (Fair Lawn, NJ, USA). Greiner 96-well black flat bottom plates (T-3021-12) for fluorescence measurements were from ISC BioExpress (Kaysville, UT, USA).

Preparation of α_1–PI solutions

(a) α_1–PI from ZLB. The concentrate of purified human α_1–PI, frozen prior to heat treatment, was thawed and dialyzed against PBS. The solution was diluted by PBS to concentration 398 μM, aliquoted and stored frozen at −70°C.

(b) α_1–PI from AR. The lyophilized powder was reconstituted in water and diluted in PBS to a concentration of ~200 μM. This stock solution was aliquoted and stored frozen at −70°C.

Concentrations of the α_1–PI diluted samples were determined spectrophotometrically using a coefficient of molar extinction $A_{280}^{0.1\%}$ 0.433 (Edelhoch 1967). The purity of α_1–PI solutions from ZLB and AR was characterized by SE-HPLC and shown to be comparable consisting of ~98% monomer and ~2% dimer. Anti-protease activity of the α_1–PI solutions were comparable within the accuracy of the in-house plate-based active site titration assay (±15%).

Preparation of RA solutions

RA stock solutions (1.28 and 2.52 mM) in absolute ethanol were freshly prepared, degassed under vacuum, purged with argon and stored at −20°C in the dark. The concentrations of RA were determined spectrophotometrically using molar extinction coefficient at λ_{max} of 49,700 M^{-1} cm^{-1} (Noy 1992).

Titration of α_1–PI with RA

Binding experiments for α_1–PI and RA were conducted according to an earlier described protocol (Karnaukhova 2007). The titration set contained 20 samples. The α_1–PI concentration was held constant (43.2 μM after aliquots of RA/ethanol were added). The amount of RA varied to give a range from ligand-to-protein molar ratio (L/P) of 0–1.92 by the step of 0.2 equivalent. The titration samples were incubated on slow rotary device at room temperature overnight in dark.

Controls

(a) A stock α_1–PI solution (43.2 μM) was used as a control for all spectral measurements.
(b) *trans*-Cinnamic acid and myristic acid were used in control binding experiments as the ligands of shorter and longer (than RA) length, respectively.

UV–vis measurements

The electronic absorption spectra were measured using an Agilent HP8453 UV-visible spectrophotometer (Agilent Technologies Deutschland GmbH, Germany) at 25 ± 0.2°C in the range 200–700 nm using a quartz cuvette with 1 cm pathlength. For the UV–vis titration series, the UV–vis spectra are presented in differential format, each after the subtraction of protein spectrum of the corresponding dilution.

Fluorescence measurements

Fluorescence measurements were carried out simultaneously using SPECTRAmax GEMINI XS Microplate Spectrofluorometer (Molecular Devices, CA, USA) at 25 ± 0.2°C. The samples of each titration set were loaded onto the Greiner 96-well black flat bottom plate using 200 μL of each sample in duplicate. The excitation wavelength was 295 nm and the emission spectra were recorded between 300 and 500 nm with maximum observed at 340 nm. The bandwidth for measuring emission was 1 nm. Fluorescence of free RA (saturated solution) in PBS, pH 7.4, was equal to the blank (buffer) solution that was subtracted. The data analysis was performed using 4.7.1 SOFTmax PRO software.

CD measurements

The visible CD spectra were recorded between 300 and 600 nm on a Jasco J-810 Spectropolarimeter (JASCO Co., Japan) at 25 ± 0.2°C in a rectangular quartz cuvette with 1 cm pathlength. The spectra were recorded with a scan speed 100 nm/min, bandwidth was 1.0 nm, and resolution was 0.2 nm. All spectra were accumulated in triplicate. Induced CD was determined as the CD of the α_1–PI/RA complex sample after subtraction of CD of the protein alone. Protein concentration in all samples was constant. An ellipticity of CD spectra is expressed in millidegrees (mdeg).

The far-UV CD spectra were measured for 10-fold diluted samples within 200–260 nm range using a quartz cuvette with 2 mm pathlength. Other experimental conditions were the same as shown above.

Native gel electrophoresis and SDS-PAGE

Native gel and SDS-PAGE analyses were performed using pre-cast 7.5 and 4–20% Tris/Gly mini-gels. SDS-PAGE was conducted under reducing conditions. SeeBlue Plus2® Prestained Standard from Invitrogen (Carlsbad, CA, USA) served as the protein ladder. Simply Blue™ SafeStain (Invitrogen) was used for staining.

Inhibitory activity assay

Evaluation of inhibitory activity of α_1–PI and α_1–PI/RA by aid of trypsin active site titration assay

Inhibitory activity of α_1–PI/RA was evaluated in comparison with initial control α_1–PI by in-house plate-based trypsin active site titration assay using BTP and chromogenic titrant NPGB (Chase and Shaw 1967). 300 μL of

43.2 μM α_1–PI or α_1–PI/RA (L/P 1.0) was loaded onto row A of 96-well plate as follows: control α_1–PI in positions A1 and A2, and α_1–PI/RA in positions A3–A6. Dilutions of α_1–PI from 43.2 to 3.8 μM were performed using multi-channel pipette to transfer 200 μL from raw A to B (and further from B to C, up to raw G) that contained 100 μL of PBS buffer; mixing was provided by three-time aspiration and release. Control row H contained 100 μL of PBS only. 100 μL of 30 μM BTP solution in 0.1 M HCl was added simultaneously to the columns 1–4, while 100 μL of PBS buffer was added to the control columns 5 and 6 (to take into account absorption of α_1–PI/RA sample upon the same dilutions). The plate was incubated on plate-reader shaker for 10 min to allow for trypsin interaction with α_1–PI. 100 μL of freshly prepared NPGB solution was added simultaneously to all wells and, after 10 s shaking on plate-reader, the end-point kinetics was recorded at 405 nm. The average absorption of each dilution in the columns A5 and A6 was subtracted from the mean values of the corresponding dilution samples of the columns A3 and A4, and plotted versus control (the corresponding mean values in columns A1 and A2).

Comparison of the inhibitory activity of α_1–PI and α_1–PI/RA by SDS-PAGE

10 μL of 43 μM of PPE in PBS was mixed with 10 μL of 43.2 μM solutions of (a) α_1–PI/RA of L/P 1.0, (b) α_1–PI, and (c) PBS. All mixtures were prepared in duplicate and incubated at room temperature for 10 min. The samples were mixed with 20 μL of the sample buffer, centrifuged at 5,000 rpm, heated at 100°C for 2 min and cooled on ice prior to loading onto the gel. After developing SDS-PAGE, the bands were quantified using FluorChem^{TM} 5500 (Alpha Innotech, San Leandro, CA) and ImageJ software.

Results

UV–vis absorption

Figure 1 illustrates the behavior of the same amount of RA in EtOH (a) and in PBS alone (b) and in the α_1–PI solution in PBS (c). An aliquot of RA (20 μL of 1.2 mM RA stock solution in EtOH) was subsequently added to the same volume (1 mL) of EtOH, PBS and 45 μM solution of α_1–PI in PBS. RA in EtOH (bold trace with the absorption maximum, λ_{max} around 350 nm) is stable over weeks when protected from light and oxygen. Addition of RA to the PBS immediately results in a hypochromic effect observed as a significant reduction in the absorption intensity and in a bathochromic (red) shift to 420–440 nm (curve 1, Fig. 1b). Further drop of the absorption intensities (curves 2–7, Fig. 1b) is accompanied by precipitation of RA from the solution. As seen in Fig. 1b, within 4 h the absorption intensity of RA in the buffer solution became incomparable with that of RA in EtOH.

When the same aliquot of RA is added to PBS containing α_1–PI (protein final concentration 43.2 μM), a different effect is observed (Fig. 1c). The spectrum recorded immediately upon adding RA (curve 1, Fig. 1c) indicates insignificant decrease of absorption intensity with ~1–2 nm hypsochromic (blue) shift, followed by the appearance of slight shoulder at 420 nm (curve 2 corresponding to 4 min)

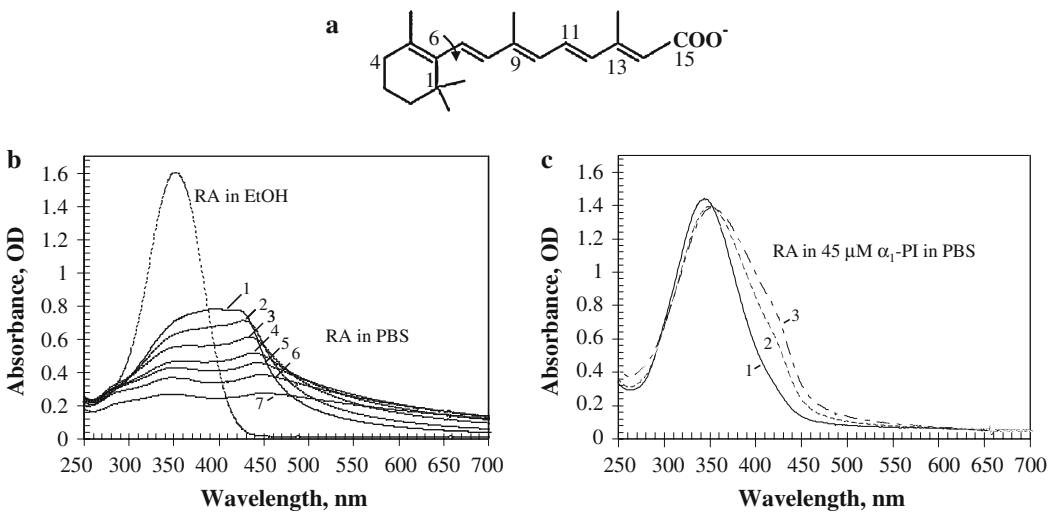

Fig. 1 **a** Chemical structure of all-*trans* RA; **b** UV/Vis monitoring of the same aliquot of RA solution added to the same volume of ethanol (*dotted trace*), and PBS (traces 1–7 corresponding to 1 min, 4 min, 12 min, 30 min, 58 min, 2 h, and 4 h, respectively), and **c** 43 μM α_1–PI solution in PBS (traces 1–3, corresponding to 15 min, 30 min, and 2 h 15 min)

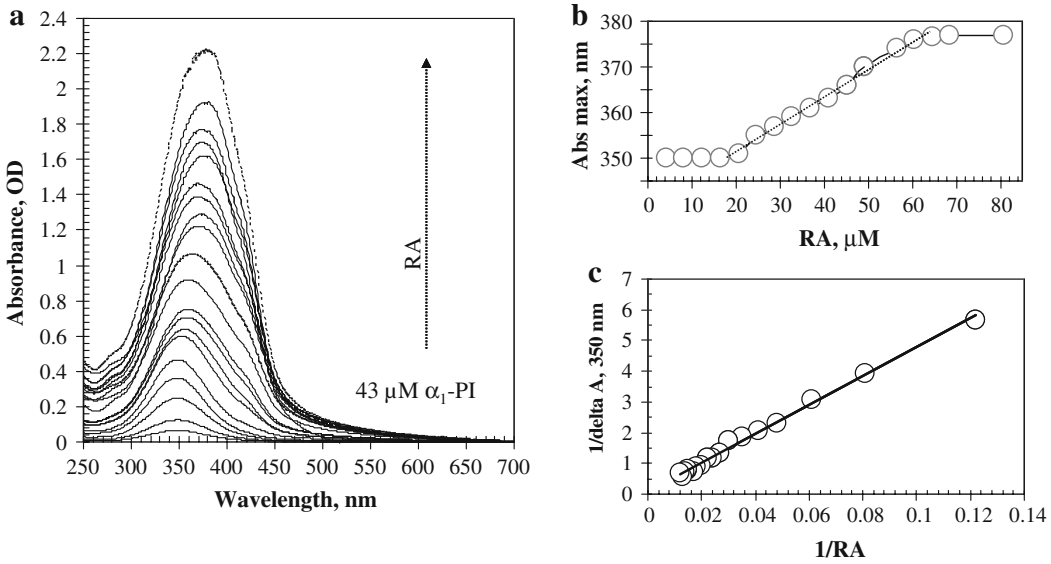

Fig. 2 UV/Vis data: **a** differential UV/Vis spectra of α_1–PI titration with RA (initial spectrum of 43 μM α_1–PI is subtracted from all spectra); **b** double reciprocal plot of change in the absorbance intensity at 350 nm as a function of RA concentration; **c** schift of the λ_{max} upon titration as a function of RA in the sample

in the final absorption spectrum with major λ_{max} at 351 nm (curve 3 corresponding to 2 h).

Figure 2 shows the differential UV–vis titration spectra that were measured after the binding reaction is completed. Up to the L/P of 0.6, the absorption spectra show a gradual increase of the regular absorption intensity at 350 nm. Starting L/P ratio of 0.6, one can notice a slight impact of the absorbance around 420 nm which, however, is almost not noticeable at higher L/P of 1.8–2.0. In the conditions used in this study, titration of α_1–PI with RA resulted in saturation at the L/P close to two moles of RA per one mole of the protein, above which the scatter made α_1–PI/RA samples turbid. Very insignificant amounts of unbound precipitated RA were determined by ethanol washes of the vials and spectral cuvette. The amount of free soluble RA is known to be below 0.2 μM, thus enabling one to conclude that up to 87 μM of RA is associated with 43 μM α_1–PI in the solution. There was a ~28 nm bathochromic shift of the λ_{max} in the differential UV–vis spectra of α_1–PI/RA titration series (Fig. 2): the absorption maximum gradually shifted from 350 nm (determined for the samples of L/P 0.1–0.6) to 378 nm (for the samples of L/P 1.5–2.0) with a linear increase of λ_{max} observed for the α_1–PI/RA samples upon the amount of RA (L/P 0.6–L/P 1.5).

Control binding experiments were performed using cinnamic acid and myristic acid in the same conditions as used for α_1–PI titration with RA. The results (unpublished) showed that cinnamic acid (a ligand of shorter than RA length) did bind to α_1–PI, whereas myristic acid (a ligand of longer than RA length) did not.

Fluorescence spectroscopy

The fluorescence quenching data further confirmed the α_1–PI/RA complexation. Titration of α_1–PI with RA results in a strong quenching of the intrinsic fluorescence of α_1–PI by the increasing content of the ligand. Figure 3 shows that a total of 50 ± 2% quenching upon protein titration with RA. Approximately 76% of the quenching effect corresponds to a high-affinity primary binding by the first equivalent of RA added (L/P from 0.1 to 0.6), followed by low affinity binding (L/P from 0.7 to 1.2 and 1.3 to 1.9). The fluorescence quenching data corresponding to the RA interval of 0–26.2 μM was exploited to calculate the apparent primary affinity constant ~48,300 M^{-1} as described elsewhere (Haugland 1996; Xie et al. 2005).

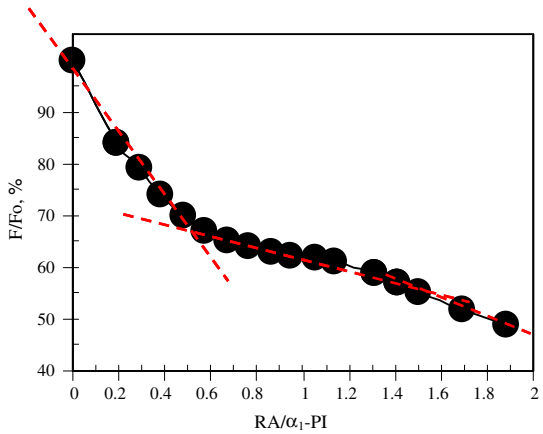

Fig. 3 Quenching of α_1–PI intrinsic fluorescence upon titration with RA

CD measurements

Whereas far-UV CD measurements did not reveal any significant alterations in α_1–PI helicity upon titration with RA, the visible CD spectra were much more informative due to a phenomenon of protein-induced chirality. Visible CD was utilized for (a) monitoring of the complex formation, (b) characterization of the titration samples, and (c) the comparison of the α_1–PI/RA complexes based on α_1–PI from various sources.

As an optically inactive molecule, RA does not show any CD either in ethanol or buffer solutions (zero CD). The host protein α_1–PI does not exhibit any CD in the visible range either, because the protein chirality is expressed in far- and near-UV range. However, adding of RA to α_1–PI solution results in the immediate response in the visible CD (Fig. 4), thus reflecting conformational distortions of the polyene chromophore by the protein environment. Since a full accommodation of the ligand requires about 3 h, the α_1–PI/RA mixed samples were incubated for 8–10 h (overnight) followed by the measurements of the end-point CD spectra. Figure 4 shows selected CD spectra of the α_1–PI titration with RA, complimentary to the corresponding UV–vis titration spectra shown in Fig. 2a. The curve corresponding to L/P of 0.2 already features almost conservative biphasic CD spectrum with the first negative Cotton effect (1st negative CE) at 400 nm and second positive CE at 335 nm with a crossover around 362 nm. The appearance of the CD in the visible region strongly indicates at the phenomenon of protein-induced chirality, thus indicating that RA binds to α_1–PI at the internal site, whereas the biphasic CD shape at this low L/P very likely suggests at least two chromophores and a possible exiton interaction. Further titration demonstrates a significant increase of the negative band with all spectra crossing close to the isobestic point and resulting in the indicative non-conservative biphasic CD.

To define whether the observed induced biphasic CD may be caused by the RA complexation with possible impurities of albumin in the α_1–PI commercial preparations (up to 6% according to the product specifications), the α_1–PI titration with RA was compared to that of human albumin (Karnaukhova 2007). Whereas the intensities of UV–vis spectra of α_1–PI/RA titration are quite close to that of albumin titration, thus indicating that a vast majority of α_1–PI molecules form complexes with RA, the CD titration data for albumin and α_1–PI are essentially different, both by the amplitude and the opposite signs of the Cotton effects. Taken together these data confirm that the observed induced CD of the RA chromophore in α_1–PI/RA series is caused by the intrinsic chirality of α_1–PI per se, not by possible albumin impurities.

A very important finding came from the CD study of α_1–PI/RA based on the RA titration of the α_1–PI preparations from different vendors. Significant conformational differences were detected in the visible CD of the same aliquots of RA complexed with different α_1–PI preparations. Figure 5 shows overlay of the CD curve shown in Fig. 4 for the sample with L/P of 1.0 (based on α_1–PI from ZLB) with the CD spectrum of the similar equimolar α_1–PI/RA complex based on α_1–PI from AR that drastically differ by the intensity. Although the crossover points are close to each other and to the absorption maxima, the amplitude is almost 20-fold more intense than that of the complex based on α_1–PI from ZLB. It is important that all the studied α_1–PI preparations complexed with RA exhibited the

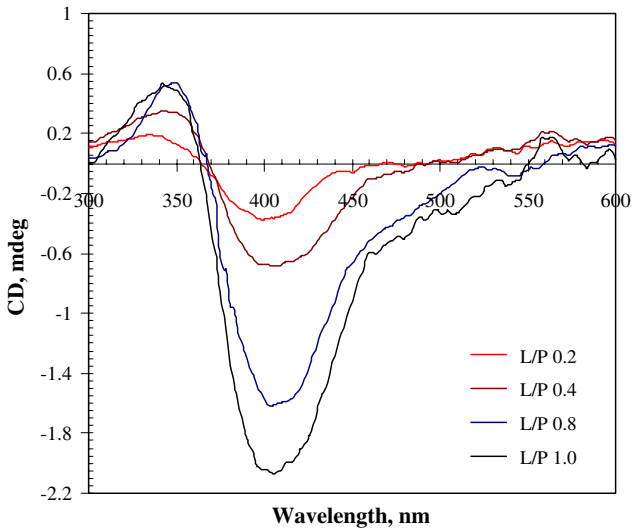

Fig. 4 CD spectra of the titration samples containing 43 μM α_1-PI (ZLB) in PBS and various amounts of RA (L/P 0.2, 0.4, 0.6, 0.8 and 1.0)

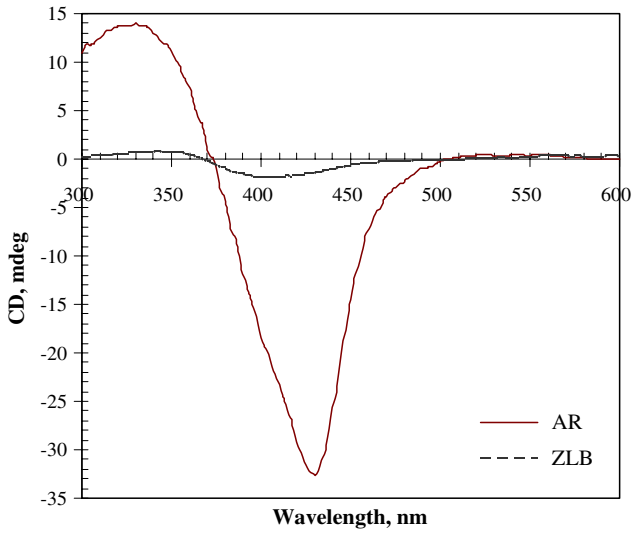

Fig. 5 CD spectra of equimolar samples of RA and α_1–PI from ZLB and AR

induced biphasic visible CD spectra, thus strongly confirming the RA complexation with α_1–PI and its inclusive nature. However, depending on the source of the α_1–PI preparations, variations in the CD shape and in the intensities of the CE's were observed.

Protease inhibitory activity of α_1–PI and α_1–PI/RA

The α_1–PI/RA inhibitory activity was evaluated by measurement of the residual protease activity of PPE and BPT after incubation with α_1–PI/RA in comparison with the control (non-complexed) α_1–PI. Figure 6a shows an active site titration plot of BPT with α_1–PI/RA of L/P 1.0 (triangles) versus control α_1–PI (circles) in the mode of "burst" kinetics using NPGB as a chromogenic titrant. The slopes (each was average of two) for the released *p*-nitrophenol plotted as a function of concentration of α_1–PI in the mixtures with trypsin are essentially the same for α_1–PI/RA (0.0067) and for control α_1–PI (0.0064), thus indicating that inhibitory activity of α_1–PI complexed with RA is essentially the same as that of the initial α_1–PI.

SDS-PAGE shown in Fig. 6b visualizes inhibitory activity of the complex α_1–PI/RA of L/P 1.0 and initial control α_1–PI towards PPE. It indicates that the α_1–PI antiprotease inhibitory activity is not affected by association with RA in case of elastase as well. Binding experiments of α_1–PI/RA and α_1–PI with protease have been assayed by the visible CD measurements to figure out whether binding with protease may force RA out from the complex with α_1–PI. When trypsin was added to α_1–PI/RA, the CD spectrum in the visible range remained unchanged, thus indicating that RA remained in the complex.

Native gel electrophoresis of α_1–PI/RA of L/P 1.0 versus control α_1–PI (not shown), suggests that the α_1–PI association with RA does not initiate, or prevent the polymerization of α_1–PI.

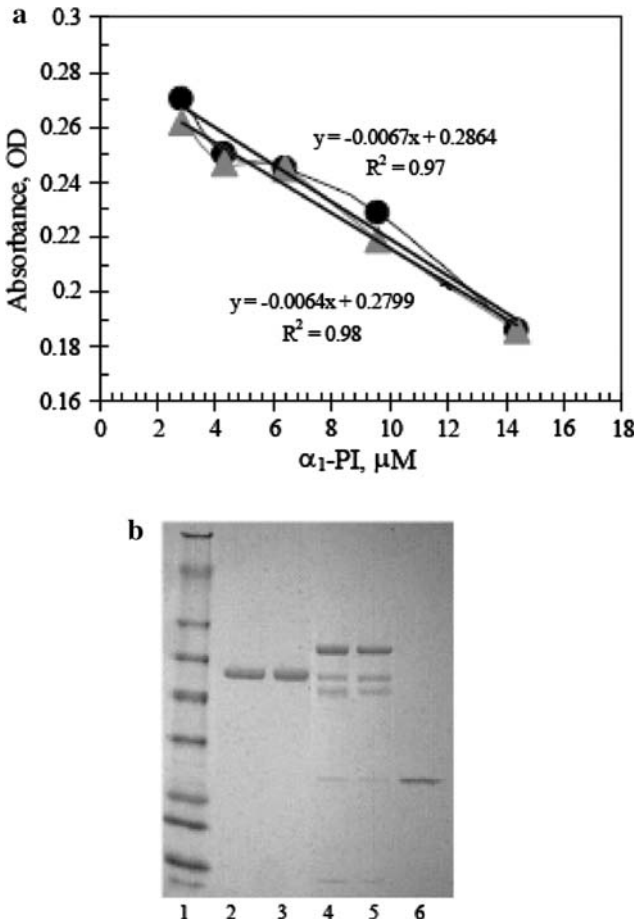

Fig. 6 Activity assessment: **a** inhibition of BPT by control α_1–PI (triangles) and the same α_1–PI complexed with RA (*circles*), each data point is an average of two measurements; **b** SDS-PAGE of α_1–PI (*lane 2*) and α_1–PI complexed with 1 mole of RA (*lane 3*), α_1–PI control mixed with PPE (*lane 4*), and α_1–PI/RA complex mixed with PPE (*lane 5*); PPE (*lane 6*); protein ladder (*lane 1*). The data are shown for α_1-PI from AR

Time-course UV–vis measurements

The UV–vis time-course measurements of the selected α_1–PI/RA complexes showed that the samples are relatively stable when stored at 4°C in dark. For instance, for the α_1–PI/RA sample of L/P 0.6: upon 1 month of storage the intensity of the absorbance at λ_{max} was still 97.5% of the initial intensity (measured at the time point 20 h), and 15 months later there was still $\sim$68% of that (spectra not shown). Although such a long storage in the solution has no practical use, these results confirm a tight association of RA with the protein. These observations are in disagreement with a relatively low affinity constant, thus suggesting that a certain stabilization of α_1–PI/RA complexes may occur in time.

Discussion

Among serpins, the affinity towards RA has been predicted and later on demonstrated for the protein C inhibitor (Huntington et al. 2003; Huntington and Liu 2009). Due to serpin structural similarities, one could assume that α_1–PI, the prototypical serpin, may also bind RA. However, due to some unique structural features of protein C inhibitor, similar interactions between α_1–PI and RA were questionable (see Huntington et al. 2003). The results presented herein provide a biophysical evaluation of the α_1–PI interactions with RA and include three types of experimental evidence (UV–vis absorption spectroscopy, fluorescence quenching spectroscopy, and induced visible CD) which indicate that α_1–PI does have an affinity to RA and can bind RA to non-covalent complexes in vitro.

Because of the extremely low solubility of RA in aqueous solutions the UV–vis studies provide the first manifestation of the RA interactions with α_1–PI. Due to a complex formation with α_1–PI: (a) the apparent solubility of RA in aqueous media is significantly enhanced, from 0.2 up to 87 μM; (b) the apparent lifetime of RA in aqueous media is significantly enhanced (up to months); (c) the observed saturation of α_1–PI solution with RA (L/P of ~1.9) suggests a stoichiometry of about two RA molecules per one molecule of α_1–PI.

Fluorescence studies provide further evidence of the complex formation between α_1–PI and RA. A strong quenching of the protein intrinsic fluorescence by the increasing content of RA strongly suggests the formation of the complex with a ligand and the proximity of the RA to one of two tryptophan residues (W^{238} and W^{194}) in α_1−PI, most likely to W^{238} that is more accessible to the quencher. The fluorescence affinity constant was evaluated as 48,300 M^{-1} which is about two orders lower than that of RA complexed with human albumin (Maiti et al. 2006). Although this affinity constant is relatively low, the long-term time-course UV–vis data suggest the α_1–PI/RA interactions might be stabilized in time.

The induced visible CD presents the strongest evidence of the complex formation and directly indicates the inclusive character of the RA binding with α_1–PI. CD provides a deeper insight into the interactions between RA and the protein. Moreover, visible CD was the method which allowed visualization of conformational differences between human α_1–PI from different sources. Induced optical activity of RA in the complexes with human α_1–PI's from various sources (ZLB, AR and CalBiochem) reflects the essential characteristics of the α_1–PI itself. The biphasic shape of the visible CD spectra, particularly observed for α_1–PI/RA samples of low L/P (e.g., 0.2), most likely suggests exciton coupling between two RA chromophores that may result from either (1) simultaneous occupation of two relatively equal α_1–PI binding sites by RA molecules, or (2) less likely, adaptation of two RA molecules within the same binding site. On the other hand, the biphasic CD may result from the overlap of two or more individual CD spectra related to the accommodation of RA in different α_1–PI species of the heterogeneous protein preparation. Regardless of the origin of the biphasic CD shape, the induced nature of the visible CD strongly supports a localization of RA inside the protein interior.

The important finding of this work was that the amplitude of the induced visible CD significantly varied depending on the α_1–PI source. The differences observed by visible CD for RA bound to α_1–PI from different sources demonstrate that α_1–PI is an intrinsically heterogeneous mixture. The α_1–PI products and preparations reflect all naturally existing variations, including a complex variably trimmed glycosylation, and also depend upon preparation procedures (e.g., Cowden et al. 2005; Kolarich et al. 2006; Weber et al. 2007). Kolarich et al. demonstrated that the α_1–PI preparations differ in molecular terms including deamidation, cysteinylation and C-terminal lysine truncation, and thus, none of the examined α_1–PI preparations is identical to native human plasma α_1–PI (Kolarich et al. 2006).

It is equally important to mention that ligand binding often reveals protein flexibility (Teague 2003). The α_1–PI conformation is intrinsically flexible, not stabilized by any internal cross-linkage, and prone to dramatic conformational changes by the definition of its function. The inherent flexibility of the α_1–PI domains and plausible variations in the loop conformations depending on the conditions and activation stages were reported by Kim et al. (2001) based on crystal structure study.

Conformationally the α_1–PI preparations are therefore quite complex mixtures, and the ligand binding reflects the co-existence of conformers and the preferences in complex formation that influence the intensity of the induced biphasic CD. In addition, as the bisignate CD indicates a possibility of the exciton interactions between two or more chromophores, it is well known that its intensity depends on several parameters, including the projection angle between the ligands (Harada and Nakanishi 1983). Thus, even small differences in the ligand local accommodation by the α_1–PIs from different vendors may result in significant differences in CD amplitude. While crystal structure of the complexes is not available, these findings emphasize the importance of the visible CD in ligand–protein study.

In summary, this study offers a novel approach to explore a ligand-binding potential of α_1–PI towards small molecules of therapeutic interest that can attain new properties to currently available α_1–PI therapies. We believe that the proposed concept should be fully investigated and further research in this direction is feasible.

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