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Reversible inactivation of serpins at acidic pH

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Abstract

The inhibitory activity of the serpins α_1-proteinase inhibitor, α_1-antichymotrypsin, α_2-antiplasmin, antithrombin and C_1-esterase inactivator is rapidly lost at pH 3 but slowly recovers at pH 7.4 with variable first-order rates ($t_{1/2} = 1.4$–19.2 min). All except α_1-antichymotrypsin undergo a variation in intrinsic fluorescence intensity upon acidification (midpoint ca. 4.5) with a slow bi-exponential return to the initial intensity at pH 7.4 (mean $t_{1/2} = 2.3$–23 min). No correlation was found between the time of fluorescence recovery and that of reactivation. The acid-treated serpins are proteolyzed at neutral pH by their target proteinases. α_1-Proteinase inhibitor was studied in more detail. Its acidification at pH 3 has a mild effect on its secondary structure, strongly disorders its tertiary structure, changes the microenvironment of Cys_{232} and causes a very fast change in ellipticity at 225 nm ($t_{1/2} = 1.6$ s). Neutralization of the acid-treated α_1-proteinase inhibitor is an exothermic phenomenon. It leads to a much faster recovery of activity ($t_{1/2} = 4 \pm 1$ min) than of fluorescence intensity ($t_{1/2} = 23 \pm 19$ min), ellipticity ($t_{1/2} = 32 \pm 4$ min) and change in total energy, indicating that the inhibitory activity of α_1-proteinase inhibitor does not require a fully native structure.

Keywords: Antitrypsin; Antithrombin; Serpin inhibition; Acidic pH

The serpins are a superfamily of widely distributed proteins that fold into a highly conserved secondary structure comprising seven to nine α-helices and three β-sheets. A large number of these proteins are serine proteinase inhibitors but inhibition of some cysteine proteinases has also been reported. In addition, some of them do not function as inhibitors but perform other roles such as hormone transport, blood pressure regulation or immunomodulation. Inhibitory serpins play important regulatory roles in blood coagulation, fibrinolysis, complement activation and inflammation [1,2].

From a kinetic viewpoint, serpins are irreversible enzyme inhibitors characterized by a second-order association rate constant [3]. Inhibition takes place in several steps. The proteinase first forms a reversible encounter complex, the so-called Michaelis complex, through binding with the serpin's reactive site loop (RSL),[1] an exposed, flexible stretch of about 17 residues eight of which (labeled P_5–P'_3) form the proteinase recognition site. The enzyme then attacks the P_1–P'_1 bond of the inhibitor which leads to a covalent ester linkage between O_γ of the active site serine residue and the carbonyl of the P_1 residue. This reaction is followed [4,5] or preceded [6,7] by translocation of the enzyme. In the final inhibitory complex the proteinase is firmly bound to the opposite pole of the serpin, the

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[1] *Abbreviations used:* RSL, reactive site loop; α_1PI, α_1-proteinase inhibitor; ACT, α_1-antichymotrypsin; AP, α_2-antiplasmin; AT, antithrombin; C1-inactivator, C_1-esterase inactivator; CD, circular dichroism; pNA, *p*-nitroanilide; Suc, *N*-succinyl; MeoSuc, methoxy-succinyl.

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P_{15}–P_1 part of the RSL inserts into β-sheet A to form the central strand of this sheet and the active site of the proteinase is distorted, thus preventing hydrolysis of the ester bond that links the enzyme to the inhibitor [8]. This important structural transition considerably stabilizes the serpin [9] and is accompanied by a large negative enthalpy change [10].

The stability of serpins against unfolding by chemical denaturants or temperature has been studied in some detail whereas the literature is poorly documented on their acid-stability. In this paper, we describe the reversible inactivation of α_1-proteinase inhibitor (α_1PI), α_1-antichymotrypsin (ACT), α_2-antiplasmin (AP), antithrombin (AT), and C_1-esterase inactivator (C1-inactivator) at acidic pH and their reactivation at neutral pH.

Materials and methods

Materials

α_1PI was purified from human plasma as described [11]. All other serpins were purchased from ART (Athens, GA). These proteins were electrophoretically homogeneous and highly active. Porcine pancreatic trypsin (EC 3.4.21.4) was from Sigma (St. Louis, MO), bovine pancreatic chymotrypsin (EC 3.4.21.1) from Worthington (Freehold, NJ) and human plasmin (EC 3.4.21.7) and neutrophil elastase (EC 3.4.21.37) from ART. Most pNA substrates came from Bachem, Bubendorf, Switzerland and were dissolved in dimethylformamide. D-Ile-Pro-Arg-pNA, also called S-2288 was from Chromogenix, Italy and was dissolved in water.

Fluorescence

We used a thermostated (25 °C) Shimadzu RF 5000 spectrofluorimeter with 1 cm path cuvettes. The influence of pH on the intrinsic fluorescence emission of serpins was monitored as follows. An aliquot of an aqueous solution of serpin was diluted into 15 mM citrate buffer pH 3–6 or in 50 mM Hepes buffer pH 7.4 and the fluorescence intensity was read after 10–15 min using $\lambda_{ex} = 280$ nm and $\lambda_{em} = 335$ nm. Before addition of the proteins, the apparatus was set to zero by reading against the buffer. The final serpin concentrations were 3.8, 1.5, 0.6, 0.2 and 1.2 μM for α_1PI, ACT, AT, AP and C1-inactivator, respectively. The fluorescence emission spectrum of α_1PI was recorded using an excitation wavelength of 280 nm. Kinetics of intrinsic fluorescence change were measured using $\lambda_{ex} = 280$ nm and $\lambda_{em} = 335$ nm. Nonlinear regression analysis was done using the Grafit software from Erithacus Software.

The α_1PI–acrylodan complex was prepared as follows. α_1PI (30 μM) was reacted for 1 h with 6 mM reduced glutathion in 20 mM Tris, pH 7.3, to free its Cys_{232} residue [12]. After chromatographic removal of glutathion using a PD 10 column (GE Healthcare, Orsay, France), the efficiency of reduction was confirmed using Ellman's method [13]. One hundred microliters of 25 mM acrylodan (Sigma, St. Louis, Mo) dissolved in dimethylformamide was then reacted for 1 h with 5 ml of 20 μM α_1PI after which the free reagent was removed by dialysis. The modified protein did no longer react with Ellman's reagent. Fluorescence emission spectra of α_1PI–acrylodan were recorded using $\lambda_{ex} = 384$ nm. The quantum yields were calculated as described by Prendergast et al. [14].

Circular dichroism

CD spectra were recorded using a thermostated (25 °C) AVIV model 62DS spectropolarimeter and 0.1 or 1 cm path quartz cells for far-UV or near UV spectra, respectively. The bandwidth of the monochromator was set to 1.5 nm. The spectrum of α_1PI dissolved in distilled water (pH 6.8) was first recorded. The inhibitor solution was then brought to pH 3.0 by addition of a small aliquot of concentrated HCl and recording was done again.

Kinetics of ellipticity change at 225 nm was studied using a MOS-400/CD optical system from Bio-Logic (Claix, France), an equipment designed for monitoring fast CD variations. The reactions were followed at 25 °C in a 1 cm path quartz cuvette inserted in a thermostated cell holder. Data recording and processing were done with the Bio-Kine software provided by the manufacturer. Acidification of α_1PI was triggered by rapidly injecting 300 μl of inhibitor dissolved in 4 mM phosphate buffer pH 6.8 into the observation cell containing 2200 μl of 5 mM citrate buffer pH 3. The ellipticity was continuously recorded at 225 nm, a wavelength at which the signal to noise level was optimal. A propeller device ensured continuous stirring of the reaction medium. This very solution was then neutralized with 70 μl of 2 M Tris buffer pH 8 and recording was re-started.

Microcalorimetry

We used a VPITC microcalorimeter from Microcal Inc., Northampton, MA. Instrument control, data acquisition and analysis were done with the VPViewer and Origin softwares provided by the manufacturer. The reference and sample cells were filled with 50 mM Hepes, 100 mM NaCl, pH 7.4 (1.4 ml each) whereas the spinning syringe was loaded with a 0.11 mM solution of α_1PI in 5 mM citrate, 100 mM NaCl, pH 3.5. The thermal power was continuously monitored. When thermal equilibrium was reached (about 15 min), the experiment was started by injecting a small volume of α_1PI solution into the sample cell.

Differential scanning calorimetry

We used a DASM-4 microcalorimeter (Poushchino, Russia). The thermograms were recorded using a 2.5 mg/ml solution of α_1PI and a heating rate of 1 °C/min. The buffers were 100 mM Hepes pH 7.4 or 100 mM acetate pH 3.3. Data analysis were done with Origin.

Thermal inactivation

Solutions of 15 μM acidified α_1PI in 15 mM citrate buffer pH 3.0 were incubated at 70 or 100 °C. After selected time intervals, 3–10 μl samples were removed and diluted into 50 mM Hepes buffer pH 7.4 containing 100 mM NaCl. These mixtures (990 μl) were incubated for 25 min at 25 °C to reactivate α_1PI and subsequently reacted with 10 μl of 8 μM chymotrypsin dissolved in the pH 7.4 buffer. Following a further 10 min incubation, the activity of free chymotrypsin was measured with 0.5 mM Suc-Ala_2-Phe-pNA. Native α_1PI was incubated in the pH 7.4 buffer and its chymotrypsin inhibitory capacity was tested directly.

Proteinase inhibitory capacity

Bovine pancreatic chymotrypsin was used to measure the inhibitory capacity of α_1PI and ACT, porcine pancreatic trypsin was used to quantitate the inhibitory activity of AT and AP and human plasmin served to measure the inhibition of C1-inactivator. Stock solutions of trypsin and chymotrypsin were made up in 1 mM HCl containing 20 mM $CaCl_2$ and plasmin was dissolved in 10 mM phosphate, 85 mM NaCl, pH 7.4. The serpins were allowed to incubate for 15 min at 25 °C in 5 mM sodium citrate/citric acid buffer of desired pH. The acidic solution of C1-inactivator was neutralized with a small aliquot of 2 M Hepes buffer pH 7.4. All other acidic solutions of serpins were diluted with large volumes of 50 mM Hepes buffer pH 7.4. The neutralized solutions were allowed to stand at 25 °C for variable periods of time before addition of small aliquots of proteinase and substrate. The residual enzymic activities were measured at 410 nm and 25 °C and analyzed using the Grafit software.

Preliminary experiments were run to find out the final concentration of proteinase in the spectrophotometric assay. This concentration had to satisfy two criteria: (i) being the minimal concentration required to fully saturate the serpin. This was determined from linear titration curves