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Tryptophan rotamers that report the conformational dynamics of proteins*

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Abstract: The binding of acetyl–pepstatin to the Q7K/L33I/L63I mutant of HIV-1 protease was studied by fluorescence, phosphorescence, and 500-ps molecular dynamics. The protease is a homodimer with two tryptophans per monomer. Maximum entropy method (MEM) analysis and acrylamide quenching results show two tryptophyl, tryptophan (Trp) populations in the apoenzyme that merge into one in the complex. These results are in agreement with molecular dynamics simulations indicative of Trp asymmetry in the apoenzyme as revealed by the occurrence of nonequivalent Trp42 indole rotamer interconversions, not observed for the complex. Analysis of the local Trp42B environments of the apoenzyme with respect to possible quencher groups shows that the χ_2 interconversions do not influence the lifetime, while the χ_1 interconversions do. Upon binding the inhibitor, Trp42B acquires a single conformation with the same lifetime and orientation as that of Trp42, and also with less quenching accessibility. Thus, protein conformational dynamics become constrained with inhibitor binding. This conclusion is supported by red-edge effect experiments and phosphorescence lifetime measurements. The low temperature τ_p (~5.8 s) is quenched to ~200 μs as protein motions become activated around the glass transition temperature. In the case of the complex, the phosphorescence lifetime data show a more cooperative activation of the quenching mechanisms.

INTRODUCTION

HIV-1 protease is a dimer (Fig. 1), consisting of two identical subunits that each contain two tryptophan (Trp) residues located at positions 6 and 42. For the most part, the protease is β-strand ordered, with very little α-helical structure (residues 86–94). Inhibitors bind at the "active site triad", consisting of Asp25, Thr26, and Gly27, located at the dimer interface. The binding event is accompanied by a large conformational change occurring in the so-called "flap region" (residues 42–58 in each monomer), which features a 4.6 Å pincer-opening motion of the flaps to accommodate the inhibitor [1]. The two types of Trp residues (6 and 42, respectively) are not equivalent: Trp6s extend out in solvent while Trp42s have their indole rings flipped back on the CA–CB bond and in closer contact with the protein matrix. The presence of these intrinsic tryptophans makes the protease a good candidate for using fluorescence and phosphorescence as dynamic probes.

In this work, we studied the Q7K/L33I/L63I mutant of HIV-1 protease, because it has been shown to resist the ubiquitous protease autoproteolysis process [4]. This made possible the first comparative study of HIV-1 protease and of its complex to an inhibitor. We describe the dynamic behavior of the Trp

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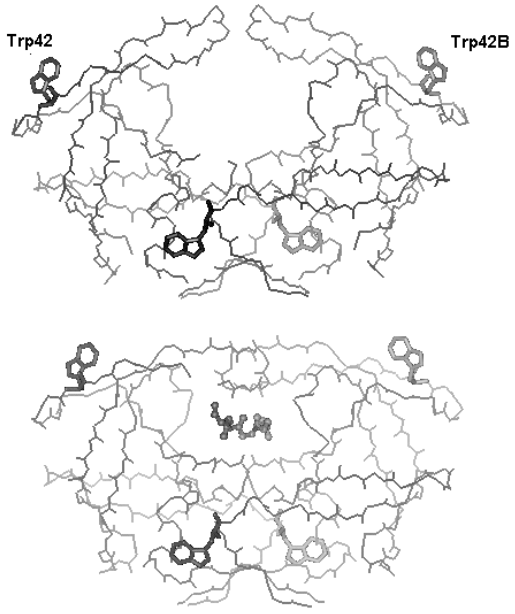

Fig. 1 Backbone rendering of HIV-1 protease (top) and of its complex with acetyl pepstatin (bottom). The Trp residues and the inhibitor are rendered as sticks. Trp 42s are located in the flexible flap region and Trp6s at the other end of the structure. Trp6s are fully solvated, this is not seen in the shown orientation. Models were generated from the following X-ray structures: 5hvp.ent [2]; 1a30.ent [3].

residues during the course of 500 ps MDS simulations and interpret the *ns*-fluorescence data on the basis of Trp42B rotamer states exploring different local quenching environments. Additionally, we show that the decay range of the phosphorescence lifetime is significantly shifted to higher temperatures in the case of the complex, thus showing a more cooperative activation of the quenching mechanisms for the enzyme-inhibitor complex. We also show that the dipole relaxation around excited-state Trps as estimated from red-edge effect experiments leads to the same conclusion, i.e., that an overall restraint of conformational dynamics characterizes the inhibitor complex relative to the unbound protease.

METHODS

Experimental

The Q7K/L33I/L63I HIV-1 protease mutant was expressed in *Escherichia coli* 1458 and purified as described elsewhere [5]. Fluorescence and phosphorescence measurements were performed at concentrations of ~11 μM in 50 mM phosphate buffer containing 50 mM disodium-hydrogen-phosphate, 1 mM EDTA, 2 mM DTE, 0.1 M NaCl, 10 v/v % glycerol, and 0.1% PEG 2000, pH = 7.5. Acetyl–pepstatin was purchased from Sigma Chemicals and dissolved in DMSO. Aliquots of 0.5 or 1.0 mM stock solutions were added to the protein solution, at a 1:2 ratio. Fluorescence/phosphorescence measurements were carried out on an Edinburgh Analytical Instruments CD900 luminometer, fitted with a Hamamatsu R955 PMT and a 75-W xenon lamp. Lifetime measurements were recorded using an nF900-type flashlamp. The decay curves were recorded using a time-correlated, single-photon counting method. The excitation wavelength was 295 nm, fluorescence was measured at 340 nm, and phosphorescence at 435 nm. Samples were deoxygenated by enzymatic oxygen digestion. Fluorescence

decay measurements were performed at LURE (Orsay) on the experimental setup installed at the SB1 window of the Synchrotron storage ring Super-ACO, which provides excitation pulses with frequency of 8.33 MHz and 500-ps pulse width. The decay data were analyzed by the method of maximum entropy at LURE [6].

Computational

The available X-ray coordinates for HIV-1 protease complexed to acetyl-pepstatin (pdb5hvp.ent, 2.0 Å [2]) and that of the Q7K/L33I/L63I mutant (pdb1a30.ent, 2.0 Å [3]) were used to generate the models, which were subjected to energy minimization using the Discover-3 module of the InsightII software package (MSI, San Diego, California) on an SGI R10000 workstation, with the extensible systematic force field (ESFF). Missing hydrogens were added subject to van der Waals constraints and consistent with the ionization state of charged R-groups at pH 7.5. To remove artifacts due to the addition of explicit hydrogens, energy minimization was performed using a conjugate gradient algorithm until the average (rms) energy derivative reached 0.1 kcal $mol^{-1}A^{-1}$. The structures were explicitly solvated in periodic boundary conditions (PBC) cell of water molecules (6888 waters), also retaining the water molecules of the X-ray structure. A dielectric constant of 1 was used throughout the simulations. Constant number of atoms, volume, and temperature (NVT) simulations were carried out at a constant temperature of 300 K using a leapfrog algorithm with a time step of 1 fs. 600-ps trajectories were acquired, and the initial equilibration 100 ps were disregarded in the analysis. Unbound interactions were taken into account using the cell multipole method [7].

RESULTS AND DISCUSSION

Both the fluorescence spectra and acrylamide quenching experiments show two differently accessible Trp populations in the apoenzyme with collisional quenching constants of 6.85×10^9 $M^{-1}s^{-1}$ and 1.88×10^9 $M^{-1}s^{-1}$ respectively, that merge into one in the complex with $k_q = 1.78 \times 10^9$ $M^{-1}s^{-1}$ (cf. Fig. 2). The fluorescence decay experiments evaluated by MEM analysis also show two lifetime components in the ns range in the decay of the apoenzyme, and one, characterized by a broader distribution in the case of the complex. We interpret these results as indicative of a Trp population capable of sensing the difference between the inhibitor-bound and unbound states of the protease.

This is supported by the results of the 500-ps molecular dynamics simulation (MDS) trajectories that are analyzed in terms of Trp indole rotamer dihedral angles. In the apoenzyme, only Trp42B is

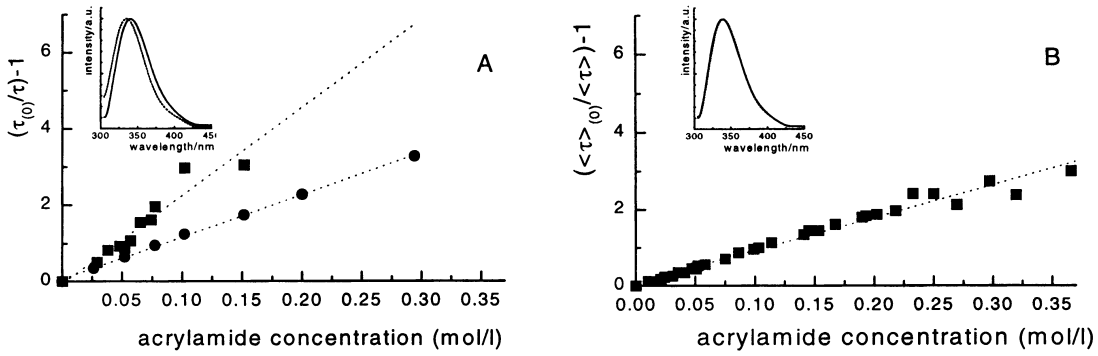

Fig. 2 Stern–Volmer plots for τ_1 (■) and τ_2 (●) discrete lifetime components of the apoenzyme (A) and for the complex (B). Insert: Fluorescence spectra at 0 M (solid line) and 0.35 M (dashed line) acrylamide concentrations, excitation wavelength: 295 nm.

interconverting between two χ_1 rotamer states (g^+/g^-), one of which comes within quenching distance of nearby Tyr59. In the other monomer, no such flipping between χ_1 rotamer states occurs, i.e., Trp42B is never brought into contact with protein matrix potential quencher groups. Upon binding the inhibitor, the previously interconverting Trp42B stops its flipping, thus becoming similar to the other, noninterconverting Trp42 (cf. Fig. 3).

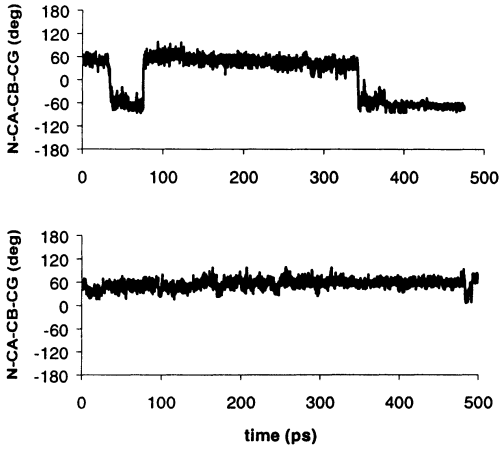

Fig. 3 Variation of the χ_1 dihedral angles of Trp42B in HIV-1 protease (top) and in the complex (bottom) during a 500-ps MDS trajectory.

The conclusion is that protein conformational dynamics around Trps 42 become constrained as an effect of binding the inhibitor. This interpretation is also supported by the results of the red-edge effect experiments that probe the dipole relaxation around the excited state Trps.

The overall effect on conformational dynamics can be assessed from the phosphorescence lifetime measurements as a function of temperature (cf. Fig. 4). The lifetime at low temperature (~5.8 s) becomes quenched to about 200 μs when the motions of protein conformation become activated around the glass transition temperature. In the case of the enzyme-inhibitor complex, the decay range of the phosphorescence lifetime is significantly shifted to higher temperatures and definitely shows a more

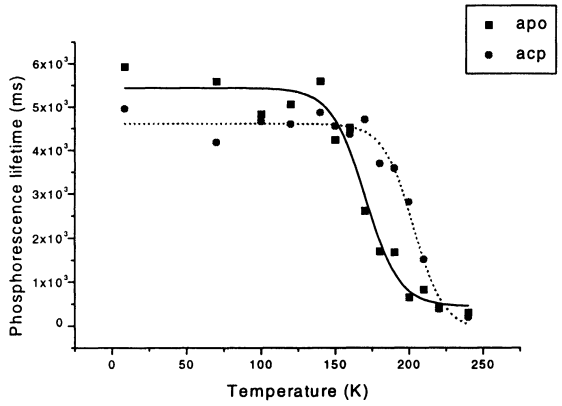

Fig. 4 Temperature dependence of the phosphorescence lifetime. Upon increasing the temperature, a characteristic shortening in the lifetime can be observed, a consequence of the increased mobility of flexible protein segments close to Trps. The shift of the glassy-fluid transition toward higher temperatures shows a more stable structure in the case of the complex.

cooperative activation of the quenching mechanisms. We thus conclude that an overall reduction of conformational dynamics characterizes the inhibitor complex, but this effect can also be well monitored by one single Trp even if the protein is relatively small and the Trp is not fully buried in the structure.

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