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Flexibility of cold- and heat-adapted subtilisin-like serine proteinases evaluated with fluorescence quenching and molecular dynamics

Ásta Rós Sigtryggsdóttir [a], Elena Papaleo [b], Sigríður H. Thorbjarnardóttir [c], Magnús M. Kristjánsson [a,*]

[a] Deparment of Biochemistry, Science Institute, University of Iceland, Reykjavík, Iceland
[b] Structural Biology and NMR Laboratory, Department of Biology, University of Copenhagen, Ole Maaloes Vej 5, DK-2200 Copenhagen, Denmark
[c] Institute of Biology, University of Iceland, Reykjavík, Iceland

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ABSTRACT

The subtilisin-like serine proteinases, VPR, from a psychrotrophic *Vibrio* species and aqualysin I (AQUI) from the thermophile *Thermus aquaticus*, are structural homologues, but differ significantly with respect to stability and catalytic properties. It has been postulated that the higher catalytic activity of cold adapted enzymes when compared to homologues from thermophiles, reflects their higher molecular flexibility. To assess a potential difference in molecular flexibility between the two homologous proteinases, we have measured their Trp fluorescence quenching by acrylamide at different temperatures. We also investigated protein dynamics of VPR and AQUI at an atomic level by molecular dynamics simulations. VPR contains four Trp residues, three of which are at corresponding sites in the structure of AQUI. To aid in the comparison, a Tyr at the fourth corresponding site in AQUI was mutated to Trp (Y191W). A lower quenching effect of acrylamide on the intrinsic fluorescence of the thermophilic AQUI_Y191W was observed at all temperatures measured (10–55 °C), suggesting that it possesses a more rigid structure than VPR. The MD analysis (Cα rmsf profiles) showed that even though VPR and AQUI have similar flexibility profiles, the cold adapted VPR displays higher flexibility in most regions of the protein structure. Some of these regions contain or are in proximity to some of the Trp residues (Trp6, Trp114 and Trp208) in the proteins. Thus, we observe an overall agreement between the fluorescence quenching data and the flexibility profiles obtained from the MD simulations to different flexibilities of specific regions in the proteins.

1. Introduction

It has been proposed that molecular mechanisms of temperature adaptation of proteins involve adjustment of molecular flexibility. Thus the higher catalytic activity, usually observed for cold adapted enzymes when compared to homologues from thermophiles, has been suggested to reflect their higher molecular flexibility [1,2]. Thermophilic enzymes are characterized by relatively rigid structures which must maintain active enzyme conformations, under temperature conditions which would lead to denaturation of homologues adapted to lower temperatures [2–8]. For cold adapted enzymes the challenge is, on the other hand, to ensure adequate molecular motions required for catalysis at low temperatures [2,3,9–15]. The increased flexibility of cold adapted enzymes need not be spread over the whole protein structure (global flexibility), but instead may be localized in smaller regions, that may affect the mobility of active site structures (local flexibility) [2,9–15]. Although different protein families appear to adopt different molecular mechanisms for the optimization of global or local flexibility at different temperatures, some structural tendencies have emerged from several comparative studies. Thus, for cold adapted enzymes, fewer or weaker noncovalent interactions, including ion pairs and hydrogen bonds, less compact hydrophobic packing, fewer prolines in loops, and more favorable surface to solvent interactions [2,13,16–19], are molecular factors which have been correlated to their temperature adaptation.

We have studied the temperature adaptation of subtilisin-like serine proteinases (subtilases) from the proteinase K family. VPR, a cold adapted subtilase from a psychrotrophic *Vibrio* sp. and aqualysin I (AQUI) from the thermophile *Thermus aquaticus*, are structurally closely related, but differ significantly with respect to thermal stability and catalytic properties, reflecting their different temperature adaptation [20,21]. Crystal structures are available for both proteinases and their stability and enzymatic properties have been well characterized, as well as those of several other related enzymes [20–26]. These subtilases thus make excellent tools for studying molecular mechanisms underlying temperature adaptation. We have used structural comparisons and site directed mutagenesis to test some hypotheses about the roles of

Abbreviations: PMSF, Phenylmethanesulfonyl fluoride; VPR, A subtilisin-like serine proteinase from a psychrotrophic *Vibrio* species; AQUI, Aqualysin I; Suc-AAPF-NH-Np, Succinyl-AlaAlaProPhe-*p*-nitroanilide; MD, Molecular dynamics; rmsf, Root mean square fluctuation

* Corresponding author at: Department of Biochemistry, Science Institute, University of Iceland, Dunhagi 3, 107 Reykjavík, Iceland. Tel.: +354 525 4800; fax: +354 552 8911.
E-mail address: mmk@hi.is (M.M. Kristjánsson).

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different residues and interactions in the temperature adaptation of these enzymes [25,27–30].

In previous studies we have observed that the reactivity of side groups of corresponding residues to chemical modification by specific reagents in the proteins has differed. For instance reactivity of their corresponding disulfides to sulfitolysis, as well as of a methionine residue, located in the active site of the enzymes, to oxidation, differed significantly [20]. The observed difference in reactivity of these groups at the corresponding locations in the two closely related enzymes most likely reflects how accessible the reactive cystine and methionine residues are to the small molecule reagents at the corresponding sites in the enzymes. The different accessibility of these groups to small molecules may, on the other hand, reflect a difference in molecular compactness, or flexibility of their protein structures [20].

In this study, we estimate the accessibility of Trp residues in the two enzymes by using fluorescence quenching. In such quenching experiments a decrease of intrinsic fluorescence of the protein resulting from collisions of a small molecule quencher (typically acrylamide) with the fluorophore, Trp, is measured. As the degree of quenching reflects how readily the quencher can penetrate the structure it can be considered as an index of protein permeability and thus a low structural resolution measure of flexibility [2]. VPR contains four Trp residues in its structure (Trp6, Trp114, Trp191 and Trp208) and AQUI contains three of those residues at corresponding sites in its structure (Trp8, Trp114 and Trp208), whereas residue 191 is a Tyr (Fig. 1). None of the Trp residues are located in close proximity to the catalytic triad residues of Asp37, His70 and Ser220 (VPR numbering) in the active site of these subtilases. For the purpose of this study, i.e. to make the two enzymes equivalent in terms of Trp content and structural positions within their structures, we produced a mutant of AQUI, where Tyr191 was changed to Trp (AQUI_Y191W). The active form of the wild type and recombinant VPR has a 15 residue long C terminal extended sequence, which is not present in the thermophilic AQUI. We have previously created a truncated form of VPR (VPRΔC) where the 15 residue extended arm was removed to simulate further the structure of the thermophilic enzyme with respect to size [30]. Thus for this comparative study we created mutants of both the cold adapted and the thermostable enzyme that were structurally equivalent both with respect to size of molecules and content and locations of Trp residues within their homologous folded structures. We report here on the characteristics of the VPRΔC and AQUI_Y191W mutants, as well as their dynamic fluorescence quenching properties to reflect on the overall conformational flexibility in the region of the Trp residues of the two extremophilic subtilases. Atomic explicit solvent 100 ns molecular dynamics (MD) simulations were also employed to complement and rationalize the information from the fluorescence experiments by providing localized flexibility profiles per residue basis over the structures of the enzymes.

2. Materials and methods

2.1. Expression and protein purification

The cloned gene for aqualysin I (AQUI) and its mutants were expressed in *E. coli* (BL21) from the pJOE expression vector [29] and the VPR gene from the pBAD TOPO expression vector (in Top10 cells), as described previously [21]. Both enzymes and mutants were purified to homogeneity by previously described protocols [20,21,29].

2.2. Site directed mutagenesis

For producing the mutant, AQUI_Y191W, the method described in the protocol for Quickchange™ Site directed Mutagenesis Kit from Stratagene was followed, using a cloned *Pfu* polymerase from Stratagene and *Dpn*I endonuclease from New England Biolabs. The following primers were used: Y191W forward: 5′-CCA GCT TCT CCA ACT GGG GTA GTT GCG TGG AC-3′; Y191W reverse: 5′-GTC CAC GCA ACT ACC CCA GTT GGA GAA GCT GG-3′. The C-terminal truncated VPR mutant (VPRΔC) was produced as described previously [30].

2.3. Kinetic and thermal stability measurements

Enzymatic activity of VPR, AQUI and mutants was assayed using Suc-AAPF-NH-Np (Sigma) as a substrate as described previously [20], using a thermoregulated Thermo Heλios α spectrometer with a Thermo Spectronic Single Cell Peltier temperature controller. Kinetic parameters for activity were determined at 25 and 40 °C for VPR and AQUI, respectively, by fitting the rate data measured at substrate concentrations between 0.05 and 1 mM, to the Michaelis–Menten equation.

Thermal stability was determined by measuring the rate of irreversible inactivation at temperatures between 88 and 96 °C in 25 mM Tris, pH 8, containing 100 mM NaCl and 1 mM $CaCl_2$ for AQUI, but at temperatures between 53 and 65 °C, in 25 mM Tris, pH 8, containing 100 mM NaCl, 1 mM EDTA and 15 mM $CaCl_2$ for VPR. Samples were heated, aliquots withdrawn at intervals and immediately assayed for remaining activity against Suc-AAPF-NH-Np as described for VPR [21]. Rate constants for thermal inactivation obtained from the first order plots were used to construct Arrhenius-plots describing the temperature dependence of these rate constants (ln k vs. 1/temperature (K)). $T_{50\%}$ values were obtained from the Arrhenius-plots as the temperature at which the rate of inactivation corresponded to 50% loss of original enzyme activity after 30 min.

For determination of melting curves, samples of VPR and its mutants were typically preheated at 40 °C [20,21], followed by inhibition to a final concentration of 1 mM phenylmethanesulfonyl fluoride (PMSF) and dialysis overnight against 25 mM glycine, pH 8.6, containing

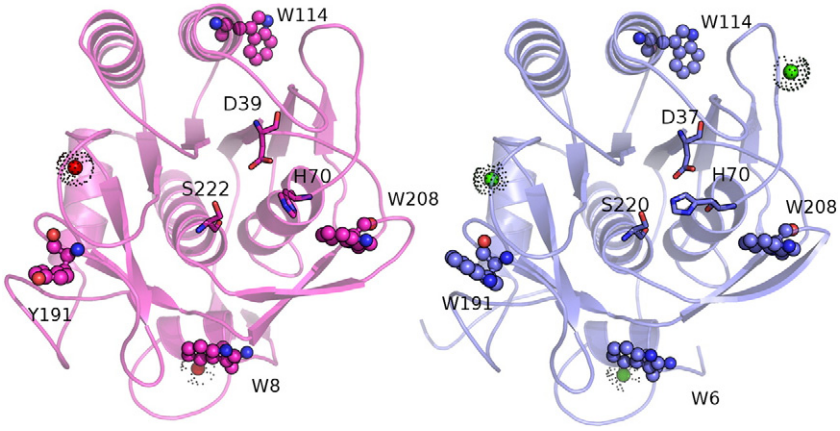

Fig. 1. Location of Trp residues in the 3D structure of AQUI (left panel) and VPR (right panel). The secondary structural elements, the calcium ions, the Trp residues and the catalytic triad (S220, H70 and D35) are shown as cartoon, spheres, sticks and ball and sticks respectively.