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Effect of Proline Substitutions on Stability and Kinetic Properties of a Cold Adapted Subtilase*

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A cold adapted subtilisin-like serine proteinase from a *Vibrio* species is two amino acids shorter at the N-terminus than related enzymes adapted to higher temperatures and has a 15 residues' C-terminal extension relative to the highly homologous thermophilic enzyme aqualysin I from *Thermus aquaticus*. These enzymes are produced as pro-enzymes with an N-terminal chaperone sequence for correct folding and a C-terminal signal peptide for secretion, which are subsequently cleaved off by autocatalysis to give the mature enzyme. A truncated form of the Vibrio proteinase where the C-terminal extension was removed and two residues near the N-terminus were substituted with proline, to resemble the N- and C-terminal regions in aqualysin I, resulted in increased thermostability and diminished catalytic efficiency. The proline substitutions shift the site of autocatalytic cleavage at the N-terminus by two amino acids, apparently by rigidifying the terminal residues and support the formation of a β-sheet that fixes the N-terminus to the main body of the protein.

Key words: kinetic properties, psychrophilic, proline, site-directed mutagenesis, stability.

Abbreviations: PMSF, phenylmethanesulfonyl fluoride; Suc-AAPF-NH-Np, Succinyl-AlaAlaProPhe-*p*-nitroanilide; VPR, a subtilisin-like serine proteinase from a psychrotrophic *Vibrio* species.

A key mechanism in the adaptation of organisms to extreme environments, *e.g.* high or low temperatures, is to adjust the properties of enzymes to function under the conditions set by their surroundings (*1*). Enzymes of thermophilic organisms are characterized by relatively rigid structures that are able to maintain active conformation under the environmental strain resulting from increased molecular motions at high temperatures. Cold-adapted enzymes, on the other hand, have to ensure sufficient molecular movements required by the catalytic mechanisms to compensate for the slow reaction rates at low temperatures (*2*). Comparative studies of amino acid contents, sequences and three-dimensional structures have not revealed any general trends applied in the molecular mechanism for different temperature adaptation. However, some tendencies have been detected towards a reduced occurrence of non-covalent intramolecular interactions, such as ion pairs and hydrogen bonds, a less compact packing of the hydrophobic core, an increased apolar surface area, longer surface loops and a reduced number of proline residues in such loops on going from higher to lower temperature adaptation (*2–4*).

Among enzymes in the proteinase K family of subtilisin-like serine proteinases are highly homologous proteins with different properties characteristic of the temperature adaptation of their host organism. Thus, a cold adapted proteinase from a psychrotrophic *Vibrio*-species, VPR, has 60% sequence identity and 76% sequence similarity to aqualysin I, AQUI, from the thermophilic bacterium *Thermus aquaticus*. The high sequence homology of these enzymes, while showing strong traits reflecting the selective pressure for increased structural stability in hot environments and sufficient catalytic efficiency in cold surroundings, makes these enzymes a feasible model for studying the molecular principles of temperature adaptation. We reported previously on mutagenic studies based on comparisons of VPR and related enzymes of higher temperature adaptation, in particular AQUI (*5*). Structural comparisons showed that AQUI has five proline residues that are not present in VPR, four of which are located in surface loops. Two of the four proline exchanges, T265P and N238P, between VPR and AQUI were considered prominent as being involved in structural stabilization of AQUI over VPR. The remaining two, N3P and I5P, located at the N-terminal of the enzyme, were not expected to affect the stability or kinetic properties of the enzymes. However, recently Sakaguchi *et al.* (*6*) reported on the reverse proline mutants in AQUI, i.e. P268T, P240N, I7P and P5N, where the substitution of prolines close to the N-terminal had the strongest effect on the properties of AQUI (*6*). These results prompted us to go back to investigate the structure of the N-terminal of VPR and related heat stable enzymes and the plausible influence of the proline exchanges between VPR and AQUI. These enzymes are produced as pro

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enzymes with an N-terminal propeptide signaling transfer over the inner cell membrane and serving as an intramolecular chaperone for correct folding and a C-terminal peptide essential for secretion of the enzyme, both of which are cleaved off by autocatalysis to form the mature enzyme. VPR also has an estimated 15 residue C-terminal extension in the mature enzyme as compared to AQUI (*7*). Furthermore, the N-terminus of the mature VPR deviates from its more stable counterparts in being two residues shorter, i.e. the site of autocatalytic cleavage at the N-terminus is translated by two amino acids. This has been confirmed with N-terminal sequencing by Edman degradation and was also unambiguous from the electron density of the crystal structure of VPR determined at 1.84 Å (*8*). In the recently determined crystal structure of a proteinase from a *Serratia* species the N-terminal residues, which are absent in VPR, form a β-sheet with residues in the main body of the enzyme (*9*). This is most likely the case for AQUI as well as related enzymes, other than VPR. The *Serratia* proteinase is highly homologous to both AQUI and VPR and although originating from a psychrophilic bacterium, it does not show characteristics typical for cold adapted proteins. Here we report on the effect of the two proline mutations at the N-terminus of VPR, the single mutation I5P and the double N3P/I5P. The mutations are made on the truncated form of VPR, VPRΔC, which lacks the 15 residue C-terminal extension in order to enhance the comparison with AQUI.

MATERIALS AND METHODS

Site-Directed Mutagenesis—Site-directed mutants were obtained with the Quickchange method (Stratagene), using a cloned *Pfu* polymerase from Stratagene and *Dpn*I endonuclease from New England Biolabs. A truncated form of VPR without the C-terminal prosequence, VPRΔC, was constructed by changing the codon TGT encoding C277 into the stop codon TAA. Two proline mutants were produced from VPRΔC, I5P, on which construct a second mutation was performed to generate a double proline mutant, N3P/I5P. The following primers were used: VPRΔCforward 5′-GCA GAC AGT GGT TAA GAG CCG GAT TGC GG-3′, VPRΔCreverse 5′-CCG CAA TCC GGC TCT TAA CCA CTG TCT GC-3′, I5Pforward 5′-GCC GTT CAA AGC AAC GCG CCG TGG GGG CTA GAC CGA ATA G-3′, I5Preverse 5′-CTA TTC GGT CTA GCC CCC ACG GCG CGT TGC TTT GAA CGG C-3′, N3P/I5Pforward 5′-CAA ATG AAG CCG TTC AAA GCC CGG CGC CGT GGG GGC TAG ACC-3′, N3P/I5Preverse 5′-GGT CTA GCC CCC ACG GCG CCG GGC TTT GAA CGG CTT CAT TTG-3′.

Expression and Protein Purification—VPR mutants were expressed with the pBAD TOPO expression vector and purified as described previously for the wild-type and recombinant VPR (*7*, *10*). Before protein measurements, samples were incubated for 30 min at 40°C, followed by dialysis against the appropriate buffer. This is a standard procedure established for the recombinant full length VPR, which is purified as a 40 kDa enzyme with the C-terminal prosequence attached. The 40°C incubation step serves to cleave off the C-terminal prosequence to ensure that there was only the 30 kDa mature enzyme present in all experiments.

Enzyme Kinetics and Thermal Stability Measurements—Enzymatic activity was assayed at 25°C with Suc-AAPF-NH-Np (Sigma) as a substrate in 100 mM Tris pH 8.6 containing 10 mM $CaCl_2$ as described previously (*10*).

Thermal stability was determined by measuring the rates of irreversible inactivation by assaying remaining activity of enzyme dissolved in 25 mM Tris, pH 8, containing 100 mM NaCl, 1 mM EDTA and 15 mM $CaCl_2$ against Suc-AAPF-NH-Np at temperatures between 55–67°C. Rate constants obtained from the first-order plots were used to construct Arrhenius plots describing the temperature dependence of these rate constants. $T_{50\%}$ 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.

Thermal unfolding transitions were measured on enzyme samples inhibited with 1 mM phenylmethanesulfonyl fluoride (PMSF) followed by dialysis in 25 mM glycine, pH 8.6, containing 100 mM NaCl and 15 mM $CaCl_2$. Measurements were carried out with a JASCO-810 Circular Dichroism spectropolarimeter equipped with PTC-423S Peltier type single cell holder by monitoring the change in ellipticity at 220 nm occurring at a constant heating range (1°C/min) from 25 to 90°C. The melting curves were normalized as described previously (*7*).

RESULTS

Kinetic Properties—The kinetic parameters of the truncated VPRΔC and VPR produced as a full-length protein are comparable. The slight decrease in k_{cat} and a higher K_m value for the truncated form are reflected in a moderately diminished catalytic efficiency, k_{cat}/K_m as measured at 25°C (Table 1). The single proline mutant, I5P, shows a significant decrease in substrate affinity with a K_m value 40% higher than VPRΔC, which is the dominating factor in the drop in catalytic efficiency. The second mutation leads to a major drop in turnover number as well as a higher K_m value relative to VPRΔC, which is the main contributor in a tenfold reduction in catalytic efficiency of N3P/I5P.

Thermal Stability—The truncated VPRΔC shows a moderate increase in thermal stability, both with respect

Table 1. **Kinetic parameters of recombinant VPR produced as full-length enzyme, the truncated form lacking 115 residues' C-terminal prosequence and the proline mutants, I5P and N3P/I5P.**

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
VPR	74.6 ± 5.0	0.166 ± 0.017	449 ± 30
VPRΔC	68.2 ± 9.9	0.184 ± 0.017	371 ± 26
I5P	58.4 ± 8.0	0.261 ± 0.018	224 ± 25
N3P/I5P	8.8 ± 1.3	0.224 ± 0.010	40 ± 7

The turnover number (k_{cat}) and Michaelis constant (K_m) were determined at 25°C in 100 mM Tris, pH 8.6, containing 10 mM $CaCl_2$ using SucAAPF-NH-Np as a substrate. The values are expressed as a mean ± SE and are based on three to six measurements.

Table 2. **Thermal stability of recombinant VPR produced as full length enzyme, the truncated form lacking the C-terminal extension, VPRΔC, and the proline mutants, I5P and N3P/I5P.**

Enzyme	$T_{50\%}$	T_m
VPR	56.0 ± 0.2	63.6 ± 0.3
VPRΔC	56.4 ± 0.1	65.2 ± 0.2
I5P	58.7 ± 0.1	67.6 ± 0.1
N3P/I5P	61.9 ± 0.2	69.3 ± 0.2

The $T_{50\%}$ values were derived from Arrhenius plots and represent the temperature at which half of the activity is lost in 30 min. T_m values were derived from melting curves determined with circular dichroism.

to $T_{50\%}$ and T_m as compared with wild-type VPR (Table 2 and Fig. 1). The difference was more distinct for T_m (~1.6°C), than for $T_{50\%}$ (~0.4°C). This might imply that the deletion of the flanking C-terminal extension in VPR makes the structure to some extent more resistant to heat denaturation but has relatively little effect on thermal inactivation. The single mutation I5P results in an increased thermal stability to a similar degree in both $T_{50\%}$ and T_m, which is further enhanced by the second proline mutation in the double mutant N3P/I5P. The total increase in stability, from wild-type VPR to the double mutant of the truncated form, N3P/I5P, is similar both regarding $T_{50\%}$ and T_m or 5.7–5.9°C. However, the effect on thermal inactivation is most pronounced upon addition of the second proline, N3P, as seen in a 3.2°C increase in $T_{50\%}$ between I5P and N3P/I5P, whereas the melting point is gradually elevated between additions of mutations.

N-Terminal Amino-Acid Sequencing—The proline substitutions studied here are located on a loop at the very N-terminus, which in AQUI, and related enzymes of greater stability than VPR seem to be fixed to the main enzyme body with a β-sheet by amino acids at the N-terminus that are absent in the mature form of VPR. The N-terminal region is highly conserved among the comparative enzymes apart from the absence of the two proline residues and the shortening of the N-terminus by two amino acids in VPR. This prompted us to hypothesize that the effect of the proline residues at that location might be to rigidify the loop resulting in formation of a β-sheet and thus shifting the site of autocatalysis at the N-terminus by two amino acids resembling the structure of the known structure of proteinase K, *Serratia* proteinase and models of AQUI. To test this, N-terminal amino acid sequencing was carried out on the double VPR proline mutant using Edman degradation. Different from the wild-type VPR with a glutamine as the N-terminal residue, the double proline mutant N3P/I5P, proved to have an N-terminus starting with the alanine in the corresponding site to the N-terminal alanine in the related enzymes (Fig. 2).

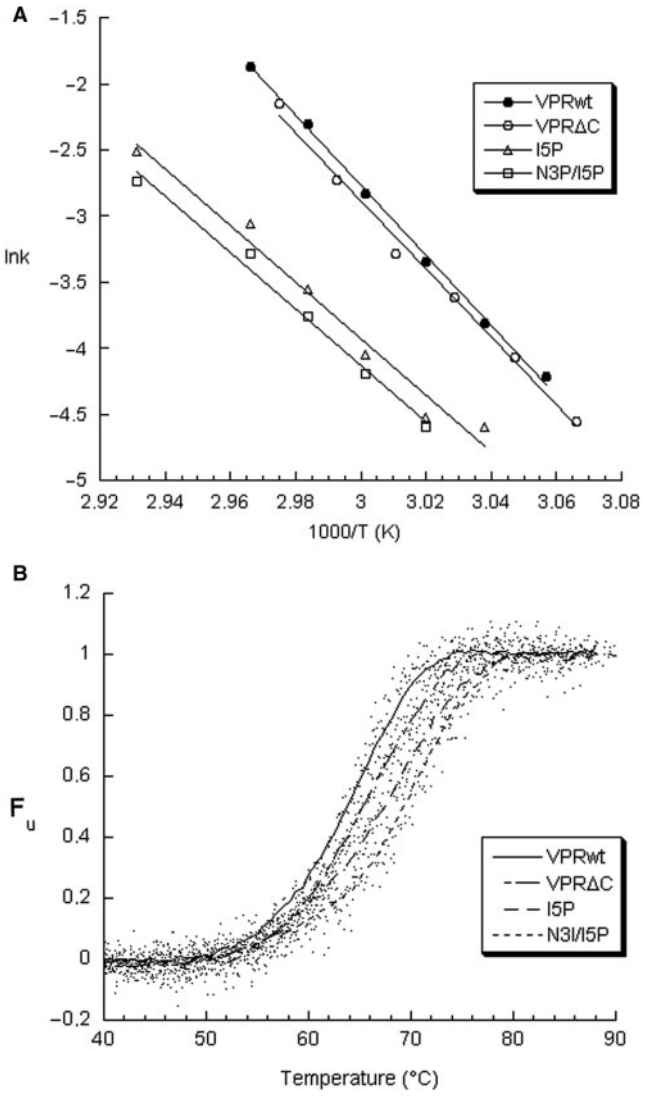

Fig. 1. **(A) Arrhenius plots of VPR and its mutants.** The rate of irreversible inactivation was determined by incubating enzyme in 25 mM Tris, pH 8, containing 100 mM NaCl, 1 mM EDTA and 15 mM $CaCl_2$ at temperatures in the range 53–64°C and measuring at time intervals the remaining proteolytic activity against Suc-AAPF-NH-Np. (B) Normalized melting curves of PMSF inhibited VPR and the mutants determined by circular dichroism. The enzyme was measured in 25 mM glycine, pH 8.6, containing 100 mM NaCl and 15 mM $CaCl_2$.

DISCUSSION

Proline residues have frequently been correlated with thermostabilization on the account of their restriction of configurations, which lowers the conformational entropy of both the folded and unfolded state, but has a stronger effect on the latter and thereby reduces the force of entropy driven unfolding (*11*). This stabilization mechanism has been suggested to be present in several thermo- and hyperthermophilic enzymes (for review see ref. *12*). Thermostabilization has been engineered in several proteins by introduction of proline residues into surface loops, β-turns, the first turn of α-helixes and at the N-cap of α-helixes (*5*, *13–15*). In our previous studies of VPR the two sites that were substituted with Pro in this study (N3 and I5), were not considered prominent candidates for thermostabilization as they were not considered to be at structurally important location with respect to thermostabilization (*5*). Interestingly, however, in the

PRK	A A Q T N A P - W G L
AQUI	A T Q S P A P - W G L
SRPK	A D Q - P S P T W G I
VPRwt	. . Q S N A I - W G L
N3P/I5P	A V Q S P A P - W G L

Fig. 2. **Alignment of the N-terminal sequence of proteinase K (PRK), aqualysin I (AQUI), the *Serratia* proteinase (SPRK), the wild-type *Vibrio* proteinase (VPRwt) and the *Vibrio* proteinase double proline mutant (N3P/I5P).** The dots in the VPR_{wt} sequence mark the sites of N-terminal residues absent in the mature enzyme.

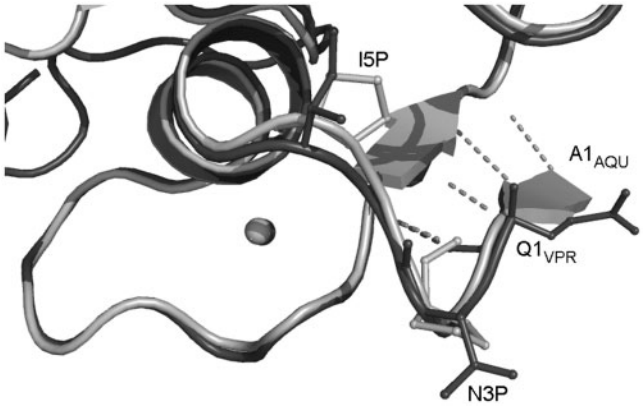

Fig. 3. **Superposition of the N-terminal regions of VPR (dark) and AQUI (light).** The N-terminal residue in the wild-type VPR, Q1, as well as the mutated residues N3, I5 and the proline residues at the corresponding sites in AQUI are shown as ball and sticks. Calcium ion is shown as a sphere. The wild-type VPR terminates with Q1, whereas the double proline mutant, N3P/IP contains two additional N-terminal residues corresponding to a β-strand in the AQUI model.

enzymes containing proline residues at these sites, they apparently mediate a tight loop structure stabilizing a β-sheet formed by the two first residues in the mature enzyme and thus anchor the N-terminus to the main body of the enzyme (Fig. 3). In wild-type VPR, the flexible loop structure without the proline residues fails to fix the N-terminus to the core enzyme. This apparently shifts the site for autocatalytic cleavage by two amino acids, which in more heat stable related enzymes participate in a β-sheet leaving a floppy N-terminal region in the cold-adapted enzyme. Loops, C- and N-termini are usually the regions of the highest thermal factor in crystal structures and are likely origins of thermal unfolding. The docking of N- and C-termini, by enhanced ion pairing or hydrogen bonds, has been reported as a stabilizing mechanism in thermophilic enzymes (*16*). In VPR the thermal factors of 3–7 amino acids segments at the N- and C-termini are 2–3-fold higher than the average thermal factors in the crystal structure, whereas in the *Serratia* proteinase only two amino acids at the N-terminus have a 50% higher thermal factor than the average. This indicates that the termini in VPR are relatively more dynamic than the rest of the structure, possibly constituting nucleation sites for unfolding. Proline in loops as well as interactions anchoring termini have been suggested as a potential structural determinant for kinetic thermostability (*17*, *18*). The stability of kinetically stable proteins is determined by unfolding kinetics rather than a thermodynamic equilibrium in favour of the native state over the unfolded state (*19*). The effect of the proline mutations at the N-terminal of VPR might indicate a kinetically dependent stability of VPR and related enzymes. Like the most investigated kinetically stable enzymes, members of the α-lytic proteinase subfamily of serine proteinases, VPR and its relatives are prosequence-dependent enzymes, i.e. their folding is mediated by a propeptide, which is subsequently cleaved off by autocatalysis. Thus, the folding and unfolding pathways are separated. The total thermostability of VPR increases to a similar degree, 5.7–5.9°C, both for $T_{50\%}$ and T_m on going from the wild-type enzyme to the double proline mutant without the C-terminal extension. However, the changes resulting from the addition of the mutations are different for these two parameters. The value of T_m rises gradually from 1.6°C upon truncation of the C-terminal extension and then by 2.4°C and 1.7°C with the addition of the prolines in the I5P and N3P/I5P mutants, respectively. The rate of thermal inactivation, which is kinetically determined, is almost unaffected by the truncation of the C-terminal extension but decreases considerably upon addition of the proline mutations, most strongly with the addition of the second mutation N3P/I5P.

Our results are consistent with those showing the importance of proline residues for the stability of AQUI (*6*), in particular for stabilizing the N-terminal region. The observed shift of the autocatalytic site in the VPR proline mutants by two residues likely facilitates the formation of a β-sheet which anchors the N-terminus to the main body of the enzyme molecule. This may lead to thermostabilization of the proline mutants as we observe in this study. We suggest that this may also be part of the different molecular mechanisms of temperature adaptation in this group of enzymes. It would be interesting to see whether the reverse mutations in AQUI, P7I and P5N, have the equivalent opposite effect, i.e. to eliminate the β-sheet fixing the N-terminus to the core enzyme.

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CONFLICT OF INTEREST

None declared.

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