NMR study of the unusually slow refolding mechanism of a protein from a hyperthermophile: NMR peak assignment and H/D exchange reaction

Satoshi Iimura¹, Taro Umezaki¹, Hiromasa Yagi², Kyoko Ogasahara², Hideo Akutsu², Yasuo Noda¹, Shin-ichi Segawa¹ and Katsuhide Yutani³

1. School of Science & Technology Kwansei Gakuin University
2. Institute for Protein Research Osaka University
3. RIKEN Spring-8 Center, Harima Institute
pyrrolidone carboxyl peptidase (PCP) from a hyperthermophile, *pyrococcus furiosus*

Number of Residues
: 208 (2 Cys at 142 and 188, Tyr, Trp)

Molecular Weight
: 22800

Molecular Form
: Homo tetramer around neutral pH
: Monomer below pH 2.5

Catalytic Function
: Removal of N-terminal pyroglutamic acid
active site residue: Cys 142

In all experiments, Cys142/188Ser mutant (PCP-0SH) was used to avoid difficulties in the analysis of refolding reactions. By this mutation, no change in 3D structure was observed.
The refolding and unfolding rates of pyrrolidone carboxyl peptidase (PCP) from *P. furiosus* are unusually slow at low temperatures and low pH\(^1\). In the refolding process of PCP-0SH (SH mutant of PCP, Cys142/188Ser), there was a burst phase from completely disordered state, D\(_2\) state, in the concentrated denaturant, to non-random denatured state, D\(_1\) state, under physiological conditions. In the transition from the D\(_1\) to the N states\(^2\), the unusually slow relaxation kinetics of PCP-0SH was apparently two state transitions and there was no stable intermediate state between them.

In order to elucidate folding mechanism of PCP-0SH at amino acid sequence level we observed H/D exchange reactions and measured 2D \(^1\)H-\(^{15}\)N HSQC spectra in the present study. NMR peak assignments of \(^1\)H-\(^{15}\)N HSQC spectra were performed by HNCACB, HN(CO)CACB, HNCO and HN(CO)CA techniques using \(^{13}\)C/\(^{15}\)N double labeled and selectively \([^{15}\)N]-Leu, -Lys, -Val-labeled PCP-0SH. Out of theoretically assignable 192 residues (16 Pro residues are included in 208 residues of PCP-0SH), 144 residues could be assigned on a \(^1\)H-\(^{15}\)N HSQC spectrum.

From real-time H/D exchange experiments in the native state, residues with elevated protection factors were found in the secondary structure region. However in presence of 2.0 M urea \(\Delta\) 5-helix in the C-terminal protrusion was less stable than other secondary structure region.

Many NMR resonance peaks in the D\(_1\) state were not observed on HSQC spectra because of the molten-globule like structure. Therefore, the progress of the H/D exchange reactions in the D\(_1\) state was presumed from \(^1\)H-\(^{15}\)N HSQC spectra after refolding. The results showed that, in the D\(_1\) state, C-terminal \(\Delta\) 6-helix already formed a native-like rigid structure, and \(\Delta\) 4-helix and the region around the V44 formed a week hydrophobic core, whereas the central \(\Delta\) \(-sheet regions were not folded.

Residual HN proton of PCP-0SH in the D₁ state on the 3D structure at pH* 3 and 4 °C.
Black; rapidly H/D exchangeable region in native structure & not assigned region
White; H/D unexchangeable region in the native structure
Red; protected region in D₁ state for limited time