Structural Investigations on the Activation of Plasminogen by Staphylokinase

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Introduction

Thromboembolic disorders have emerged to be one main cause of mortality in the western world. In order to dissolve the fibrin clots by the protease plasmin, an activation of plasminogen has to take place (1). The 15.5 kDa bacterial protein staphylokinase (Sak) isolated from Staphylococcus aureus, a non-enzymatic cofactor like streptokinase (2), forms a stoichiometric protein-protein complex with plasminogen(ogen). Cleavage of the peptide bond Lys10-Lys11 of Sak in this complex is the trigger for the formation of an enzyme species capable of activating plasminogen (3). This is in contrast to enzymatic cofactors like tissue plasminogen activator or urokinase, which activate plasminogen by their inherent proteolytic activity (4, 5). The Sak-plasminogen complex shows a high degree of specificity for cleavage of blood clots with reduced side effects because, in contrast to streptokinase activation, the Sak-plasminogen complex is strongly inhibited by α2-antiplasmin circulating in the blood (6,7,8). The protein staphylokinase is currently undergoing clinical trials for the therapy of myocardial infarction (6,9) and peripheral thrombosis (10) because of its profibrinolytic properties.

We have recently determined the NMR solution structure of the full-length protein by multidimensional heteronuclear NMR spectroscopy (11,12). Staphylokinase exhibits a well defined global structure of the β-grasp-like fold. A single central α-helix of 13 residues is flanked by a two-stranded β-sheet, both of which are located above a five-stranded β-sheet. The N-terminus folds back onto the protein core. During activation of plasminogen the N-terminal sequence of ten residues is proteolytically cleaved, thereby creating a new charged N-terminus. NMR data on this N-terminal processed variant SakAN10 was additionally acquired allowing to discuss the structure and dynamics during the initial activation step. The investigation of the conformations of native and mutant staphylokinases serves as a starting point for understanding the changes in the enzymatic specificity of plasmin and the mechanisms of plasminogen activation and will allow the design of improved drugs for lysis of blood clots.

Materials and Methods

NMR samples of staphylokinase were expressed in E. coli TG1 transformed with plasmid pMEX602sakB and purified as recently described (11,13). The concentration of the Sak sample was 1.1 mM (15N Sak). N-terminal processed, 15N-labeled staphylokinase (15N SakAN10) was obtained at a concentration of 1.3 mM after treatment of 15N-labeled Sak with plasminogen immobilized on a CNBr-Sepharose CL-4B matrix.

NMR spectra were recorded at 300 K on a Varian INOVA 750 MHz four channel NMR spectrometer equipped with pulse field gradient accessories and a triple resonance probe with an actively shielded Z gradient coil. The NMR data was analyzed with the program XEASY (14) on Silicon Graphics INDY & INDIGO2 workstations. Chemical shifts were referenced as described previously (12). Unless indicated, the 1H and 15N carriers were set at 4.74 ppm and 119.5 ppm respectively. For both samples a 15N-edited HSQC and a 3D 15N-1H Spectroscopy with increased spectral assignments. Most of the differences in the chemical shift values lie within the limits defined by the spectral resolution. While the complete assignment of the HSQC cross peaks in Sak has already been reported (11,12) in Figure 1 (left panel) only those peaks experiencing minor chemical shift changes in SakAN10 are indicated in addition to a few N-terminal residues. The removal of the first ten residues unavoidably disturbs chemical shifts in the residual N-terminal section between Asp13 and Tyr24. Only for residues Val45 and Glu65 a consistent difference in both 15N and 1H chemical shifts could be observed. In addition, protons of residues Tyr63, Leu68, Tyr73 and Glu75 in the α-helix and the adjacent loop are shifted besides Asp115, Ile120 and Lys121. The latter reside in a loop before a β-strand and
come close to each other (12).

$^{15}$N-$^1$H heteronuclear Overhauser enhancement experiments were performed to obtain an overview on possible changes in the dynamical features. This data, presented in Figure 2, reveals no major dynamical differences of the two species.

The characteristic proton-proton NOE pattern in the $\alpha$-helical and $\beta$-sheet regions seen in full-length Sak (11) have...
also been observed in the $^{15}$N-edited 3D NOESY-HSQC of the N-terminal processed Sak. Figure 3 displays a part of the helical NOE connectivities in Sak$\Delta N10$. But for the occurrence of an additional cross peak between the amide protons of Val27 and Val45 located in strands I and II (for numbering of the strands see ref. 11), the paucity of NOE contacts for the bulge region between strands II and III is also noticed in Sak$\Delta N10$ (data not shown).

The essential activation step in the proteolytic processing of Sak is the cleavage of the conformationally labile N-terminal residues Ser1-Lys10 involved in interactions with residues 40-46. The similarities in the chemical shifts, dynamical features as judged by heteronuclear NOEs and identical NOE pattern observed in Sak and Sak$\Delta N10$ indicate almost identical overall solution structural characteristics of the two species. The global fold of Sak$\Delta N10$ is also similar to the recently published X-ray structure of a proteolytically degraded Sak (18; residues 16-136), although a detailed analysis of structural differences will need a $^{13}$C,$^{15}$N-labeled sample.

The removal of residues 1-10 allows access to the bulge residues Leu40-Glu46 in a direction parallel to the $\alpha$-helix axis with an increase of the mean solvent accessible surface by more than a factor of 2 as deduced from molecular modeling (12). Two of three mutation sites (Pro48 and Ala67) identified by the alanine scan (19) lead to inactive mutants with impaired plasminogen binding. Other single site mutants of staphylokinase (20) showed that charged residues around position 68 of the helix and an intact C-terminal structure were necessary for activation of plasminogen. Exactly in these region around amino acids 48 and 67 the residues with the highest chemical shift differences between full-length and N-terminal shortened staphylokinase are observed. Both of the sites are shielded from surface exposure by the flexible N-terminal region of the staphylokinase structure and show NOE contacts to that region. The third site identified by the alanine scan covered the cleavage site at Lys10 and led to a Sak mutant which retained binding, but was incapable of activation. This emphasizes the importance of demasking a lysine residue by the cleavage between Lys10-Lys11 while the global 3D structure remains virtually unchanged.

Further work focussing on the plasmin-bound solution structure of staphylokinase is in progress.

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References


Figure 3: Strip plots of the $^{15}$N-edited NOESY-HSQC of Sak$\Delta N10$. $\alpha$-Helical NOE connectivities seen between Glu58 and Trp66 are also indicated. Peaks only present at a lower threshold are marked by boxes.


