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ARTICLE *in* ANALYTICAL CHEMISTRY · DECEMBER 2011

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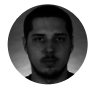

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Chemical Cross-Linking and H/D Exchange for Fast Refinement of Protein Crystal Structure

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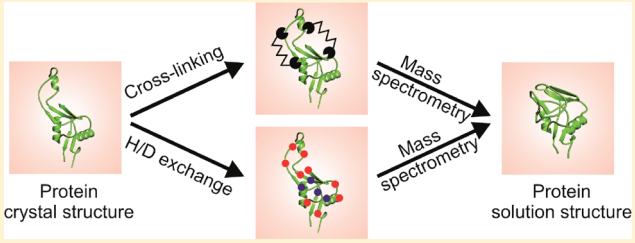
Supporting Information

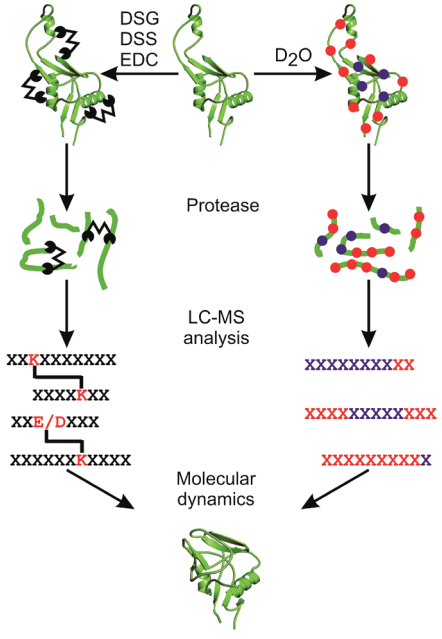
ABSTRACT: A combination of chemical cross-linking and hydrogen−deuterium exchange coupled to high resolution mass spectrometry was used to describe structural differences of NKR-P1A receptor. The loop region extended from the compact core in the crystal structure was found to be closely attached to the protein core in solution. Our approach has potential to refine protein structures in solution within a few days and has very low sample consumption.



Determining a protein structure is a prerequisite for fully understanding its function. The three-dimensional structure of proteins has been traditionally solved by X-ray crystallography or NMR spectroscopy. These techniques provide high resolution atomic data on proteins and their complexes but have some notable limitations. X-ray crystallography requires diffraction-quality protein crystals, and the structure determined by this method may be influenced by the crystal lattice. Furthermore, the crystal structure provides only a static picture of a protein under nonphysiological conditions. In contrast to X-ray crystallography, NMR spectroscopy affords dynamic structural information in solution, as well as thermodynamic and kinetic information about a protein structure. On the other hand, this method is currently restricted to proteins of molecular weight not exceeding 50 kDa. Moreover, both X-ray crystallography and NMR spectroscopy require large amounts of pure protein and are laborious.

One of the challenges of current structural proteomics is to implement high-throughput and robust techniques to probe protein structure. Mass spectrometry has become a key player in high-throughput proteomics.[1] In a perspective of *Nature Methods* in 2008, Albert Heck[2] presented an idea that future native mass spectrometry studies should focus more on the structure, topology, and dynamics of interesting proteins and protein complexes. Here, we introduce a combination of chemical cross-linking, hydrogen/deuterium (H/D) exchange and high resolution mass spectrometry to elucidate structural details of the receptor NKR-P1A (CD161a) (Scheme 1). This protein is an important activating receptor expressed at the surface of natural killer (NK) cells that act against virally infected and tumor cells.[3] The recently determined crystal structure of the extracellular domain of NKR-P1A[4] raises questions about the unique conformation of the loop region containing residues T159-D187.

Scheme 1. General Strategies for Protein Structure Characterization by Chemical Cross-Linking (Left) and H/D Exchange (Right) Coupled to Mass Spectrometry



In a homology model[5] as well as in structures of homologous proteins, this evolutionarily conserved loop is in close proximity to the compact core. Surprisingly, this loop was found to be

Received: October 25, 2011
Accepted: December 15, 2011
Published: December 15, 2011

extended from the compact core in the crystal structure of NKR-P1A (Figure 1a) and bound to a symmetry-related

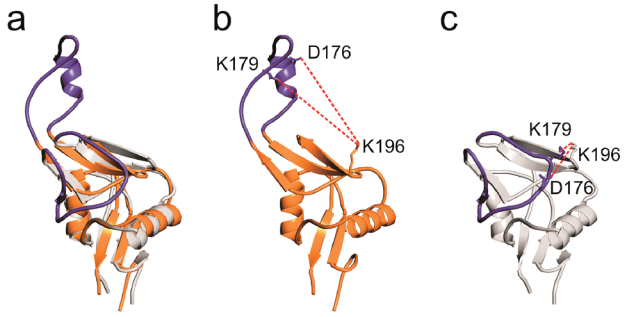


Figure 1. Cross-linking of NKR-P1A combined with mass spectrometry. (a) The different conformations of the loop region (purple) in a structural alignment of the crystal structure (orange) and the homology model (gray) of NKR-P1A. (b) The spatial distances (red dashed lines) between K179-K196 and D176-K196 in the crystal structure of NKR-P1A are inconsistent with cross-linker lengths. (c) The spatial distance constraints for K179-K196 and D176-K196 obtained in this study are consistent with the inter-residue distances in the homology model.

molecule via the domain swapping effect. Therefore, we applied mass spectrometry based techniques to investigate the dynamics and topology of this loop in NKR-P1A under near-physiological conditions.

In the first approach, NKR-P1A was subjected to chemical cross-linking.[6−8] The presence of 23 carboxyl groups and 10 primary amines in NKR-P1A provides a good basis for structural studies using this methodology. Because the high concentration of cross-linker may result in structural artifacts, it was essential to optimize molar ratio between the protein and the cross-linker. NKR-P1A was cross-linked with the zero-length cross-linker EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide, carboxyl-amine coupling, protein/cross-linker ratios 1:5, 1:10, 1:25, 1:100) and homobifunctional cross-linkers DSS and DSG (disuccinimidyl suberate and disuccinimidyl glutarate, amine−amine coupling, protein:cross-linker ratios 1:1, 1:3, 1:10, 1:20). Optimal results were obtained with 10× molar excess of EDC or 3× molar excess of DSS and DSG to NKR-P1A (0.2 mg/mL). Mass spectra of crude reaction mixtures showed that these conditions gave the highest relative yield of protein containing only a single intramolecular cross-link (data not shown), which minimized the likelihood of distorting the tertiary structure. The products obtained from chemical cross-linking were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and digested in gel by endoproteinases Asp-N, Glu-C, or trypsin. Proteolytic peptides were separated by a μHPLC system coupled online to an electrospray ionization Fourier transform ion cyclotron resonance (ESI FTICR) mass spectrometer. The high mass accuracy (below 2 ppm) was sufficient to provide unambiguous assignments of cross-links using MSlinks algorithm (http://ms3d.org).[6] The assignment of a cross-link was accepted only if the corresponding cross-linked peptides were generated by at least two out of three endoproteinases. Protein sequence was completely covered, and all cross-linked residues derived from the identified cross-linked peptides are listed in Table 1. For example, the signal at m/z 2506.2317 (error of 1.2 ppm) was assigned as a cross-link between lysines K166-K179 in peptides D162-S175/D176-G182 (Figure S1, Supporting Information). Out of 17 cross-linked residues identified, 6 were formed with EDC, 6 with DSG, and 5 with DSS.

Table 1. Summary of the Distance Constraints Derived from Structural Models and Comparison with the Cross-Link Distance Constraints

cross-linker	cross-linked residues	cross-link distance constraint (Å)	Cα−Cα distance (Å) crystal structure	Cα−Cα distance (Å) homology model
DSG/DSS	K166-K179	≤20 or ≤24	14.2	14.7
DSG/DSS	K179-K196	≤20 or ≤24	29.6	7.9
DSG/DSS	K148-K196	≤20 or ≤24	10.1	11.5
DSG/DSS	K146-K196	≤20 or ≤24	15.1	11.4
DSG/DSS	K125-K212	≤20 or ≤24	9.1	8.0
DSG	K146-K148	≤20	5.9	5.8
EDC	E147-K148	≤12	3.9	3.9
EDC	D121-K212	≤12	12.9	12.1
EDC	D123-K125	≤12	5.3	5.4
EDC	D143-K146	≤12	5.2	5.4
EDC	D176-K196	≤12	32.9	11.6
EDC	D176-K179	≤12	5.5	6.3

To compare the experimental inter-residue distances with the distances in the NKR-P1A crystal structure and a homology model, we used constraints based on the length of a spacer arm that is 11.4 Å for DSS, 7.7 Å for DSG, and 0 Å for EDC. Because the side chains of lysines and acidic amino acids have intrinsic flexibility, the following cutoffs are widely used: 24 Å between the α-carbons of lysines cross-linked with DSS, 20 Å with DSG, and 12 Å between the α-carbons of lysine and glutamic or aspartic acid cross-linked with EDC.[9] The Cα−Cα distances between cross-linked residues in the crystal structure are summarized and compared with Cα−Cα distances in a homology model[5] in Table 1. The inter-residue distance constraints obtained in this study are in excellent agreement with inter-residue distances in the crystal structure with the exception of K179-K196 and D176-K196 (Figure 1b). The distance between DSG cross-linked K179 from the loop region and K196 from the compact core of NKR-P1A should be ≤20 Å. Furthermore, the 32.9 Å distance between D176 from the loop region and K196 from the core in the crystal structure is inconsistent with the ability of EDC to cross-link these amino acids at a distance ≤10 Å. On the contrary, all experimental distance constraints fit the distances in the homology model of NKR-P1A (Figure 1c). However, this strategy does not reflect the steric repulsion and conformational changes in the protein backbone. In order to explore the spatial arrangement of selected residues involved in cross-linking, we performed a molecular dynamics simulation for the homology model of NKR-P1A. The minimal distance found during the 500 ns simulation between Nζ- Nζ atoms of K179-K196 is 6.1 Å, and the minimal distance between Cγ-Nζ atoms of D176-K196 is 2.6 Å. The distances predicted by molecular dynamic simulation for homology model of NKR-P1A are in agreement

dx.doi.org/10.1021/ac202818m | Anal. Chem. 2012, 84, 867−870

with the cross-linking distance constraints and are inconsistent with the crystal structure. Taken together, the experimental data from the cross-linking study show that in solution the loop region of NKR-P1A is not extended from the compact core, as observed in the crystal structure, but is in close proximity to the core as suggested by the homology model. Further, the absence of cross-links between K179 or D176 and lysines or acidic residues, which are spread throughout the core of NKR-P1A, suggests that the backbone in the loop region is not particularly flexible.

In the second approach, H/D exchange combined with mass spectrometry was applied to study the NKR-P1A loop conformation. H/D exchange provides information on the local solvent accessibility of proteins that is valuable in studying protein dynamics, structure, and function.[10] The aim of this analysis was to compare the kinetics of H/D exchange for NKR-P1A and NKR-P1A in which the loop was removed and replaced with two alanines. This replacement was proposed by molecular dynamic simulation as the most stable mutant since the distance of the loop termini corresponds to the distance of two amino acid residues. The expression construct for nonloop NKR-P1A (NKR-P1A^{NL}) was made by crossover PCR mediated deletion−insertion mutagenesis (Figure S2, Supporting Information). NKR-P1A and NKR-P1A^{NL} were subjected to H/D exchange followed by reduction of disulfide bonds with tris(2-carboxyethyl)phosphine (TCEP). Reduced samples were loaded onto a semiautomated chromatographic system for pepsin digestion, peptide desalting (MacroTrap), and peptide separation on a reverse phased column. The extent of deuterium incorporation into each peptide was analyzed by high-resolution ESI FTICR mass spectrometry. Pepsin digestion of proteins resulted in full sequence coverage with peptic fragments approximately 5−30 residues in length (Figure S3, Supporting Information). The mass increase reflecting deuterium incorporation (Figure 2a) was followed for all identified peptides and exchange kinetics were plotted (Figure S4, Supporting Information). Slower deuteration rates for regions 130−140, 138−143, and 191−199 were observed in NKR-P1A when compared to NKR-P1A^{NL}. This analysis suggests that these regions are protected by the loop (Figure 2b), which is consistent with the cross-linking study and the homology model but not with the crystal structure.

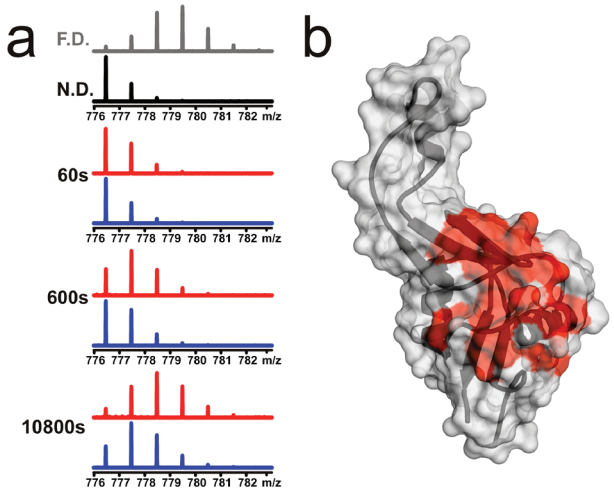


Figure 2. H/D exchange measured by mass spectrometry. (a) Changes in the isotopic pattern of doubly charged peptide 138−143 derived from NKR-P1A (blue) and NKR-P1A^{NL} (red). Three different time points are shown. N.D. represents the isotopic profile of non-deuterated peptide; F.D. represents the isotopic profile of fully deuterated peptide. (b) Differences in amide hydrogen exchange rates between NKR-P1A and NKR-P1A^{NL} mapped onto the crystal structure of NKR-P1A. The segments with a decreased exchange rate in NKR-P1A are in red.

In order to evaluate our approach, we investigated the loop conformation of NKR-P1A by NMR spectroscopy. In the 13C-edited NOESY spectra, several cross-peaks corresponding to distances ≤5 Å between the loop region and the compact core were identified (Figure 3). Due to the unique chemical shifts of some resonances, five distances were assigned unambiguously:

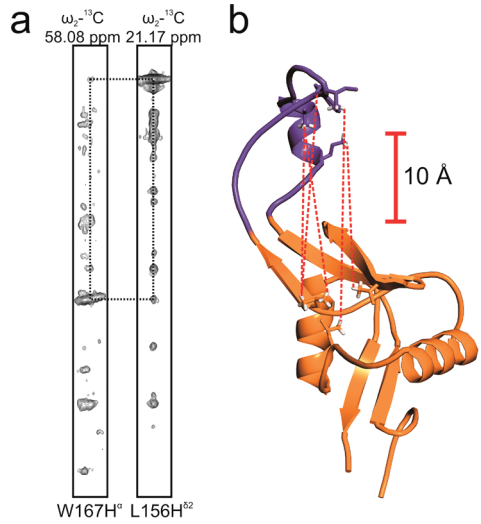


Figure 3. Conformational NMR analysis of the conserved loop of NKR-P1A. (a) Assigned cross-peaks W167Hα-L156Hδ2 and L156Hδ2- W167Hα shown in strips from a 13C-edited NOESY spectrum. (b) The distance constraints (up to 5 Å) derived from 13C-edited NOESY spectra between nearby hydrogen atoms in NKR-P1A are inconsistent with distances in the crystal structure and indicate that the loop is closely associated with compact core and association also revealed by chemical cross-linking and H/D exchange experiments.

V197Hγ2 I180Hδ1, W167Hα L156Hδ2, I168Hγ2 L129Hδ2, L173Hδ2 L156Hδ1, and L173Hδ2 L156Hδ2. These distances corroborate inter-residue distances derived from the homology model as well as data from chemical cross-linking and H/D exchange.

The approach of chemical cross-linking and H/D exchange combined with high resolution mass spectrometry described here enabled the study of structural details of the receptor NKR-P1A in solution. It revealed that the solution structure differs from the crystal structure in the conformation of the conserved loop. While the conserved loop is in close proximity to compact core in solution, it is extended from the core in the crystal structure where it interacts with the surface of a symmetry-related molecule. This suggests a testable hypothesis that the conformational change of this loop may be induced by ligand binding.

We showed that chemical cross-linking and H/D exchange coupled to high-resolution mass spectrometry provides rapid refinement of protein structure in solution. The protocol is very fast (few days), has low sample consumption (few hundreds of micrograms), and has virtually no limitations in terms of protein size.

dx.doi.org/10.1021/ac202818m | *Anal. Chem.* 2012, 84, 867−870

■ ASSOCIATED CONTENT

Supporting Information

Detailed description of experimental methods for chemical cross-linking, H/D exchange, crossover PCR-mediated deletion−insertion mutagenesis, NMR analysis, and molecular dynamics calculation, integrated with figures showing representative MS analysis of cross-linked peptides, strategy of crossover mediated PCR deletion-insertion mutagenesis, sequence coverage after pepsin digestion and plots of deuterium incorporation. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

The manuscript was written through contributions of all authors. We thank Zofie Sovova and Rudiger Ettrich for helping with the design of NKR-P1A^{NL}, Frantisek Vostarek for NKR-P1A^{NL} plasmid construction, and Mark Patchett and Vladimir Havlicek for editing the manuscript. This study was supported by grants from the Grant Agency of the Czech Republic (P207/10/1040, 303/09/0477 and 305/09/H008), the Ministry of Education, Youth and Sports of the Czech Republic (LC545), the Academy of Sciences of the Czech Republic (Institutional Research Concept AV0Z50200510 and AV0Z50520701) and from the Grant Agency of Charles University (403211/2011 and Project UNCE).

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