Communication

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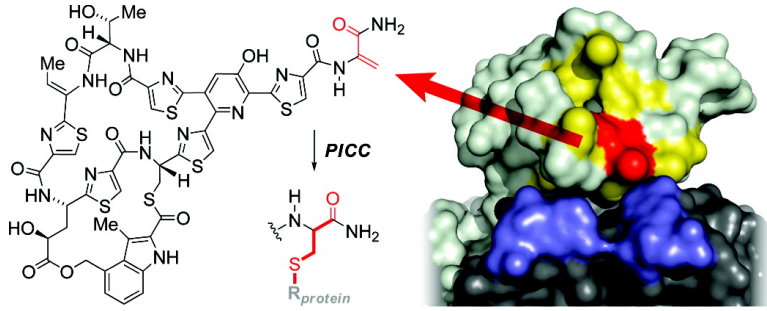

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Mapping the Binding Site of Thiopeptide Antibiotics by Proximity-Induced Covalent Capture

Sascha Baumann, Sebastian Schoof, Surendra D. Harkal, and Hans-Dieter Arndt*

Fakultät Chemie, Technische Universität Dortmund, Otto-Hahn-Str. 6, D-44221 Dortmund, Germany, and Max-Planck-Institut für molekulare Physiologie, Otto-Hahn-Str. 11, D-44227 Dortmund, Germany

Received November 30, 2007; E-mail: hans-dieter.arndt@mpi-dortmund.mpg.de

One of the major challenges in chemical biology is the characterization of small molecule ligands able to bind to and modulate larger surfaces of biomacromolecules.[1] A fruitful approach to identify and study such interactions exploits chemical reactivity conditional on proximity. Such "affinity labeling" has been widely used to survey the interior of protein receptors,[2] and mechanism-based probes are well-established for studying active sites in enzymes.[3] On protein exteriors, Wells et al. have pioneered disulfide tethers,[4] and studies of protein-surface attaching ligands have been disclosed.[5] Transferring these principles to regulatory or processive DNA–protein and RNA−protein complexes would be a significant advancement.

A composite oligonucleotide/protein surface site of this kind needs reactive functionality—most simply engineered onto the protein—to allow the conditional formation of a covalent bond with a reactive ligand.[3d] The occurrence of a proximity induced covalent capture (PICC) would then report on the bound ("active") conformation of a multimeric ligand/biomacromolecule complex (Figure 1). Such a technique could sketch geometries of unknown binding sites and by extension be valuable for identifying novel ligands. Suitable reactive group combinations might be thiol and Michael acceptor pairings.[5a]

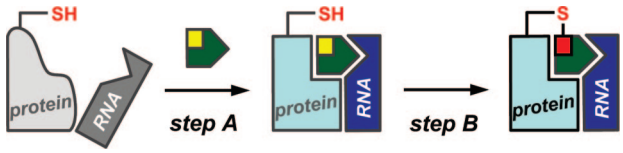
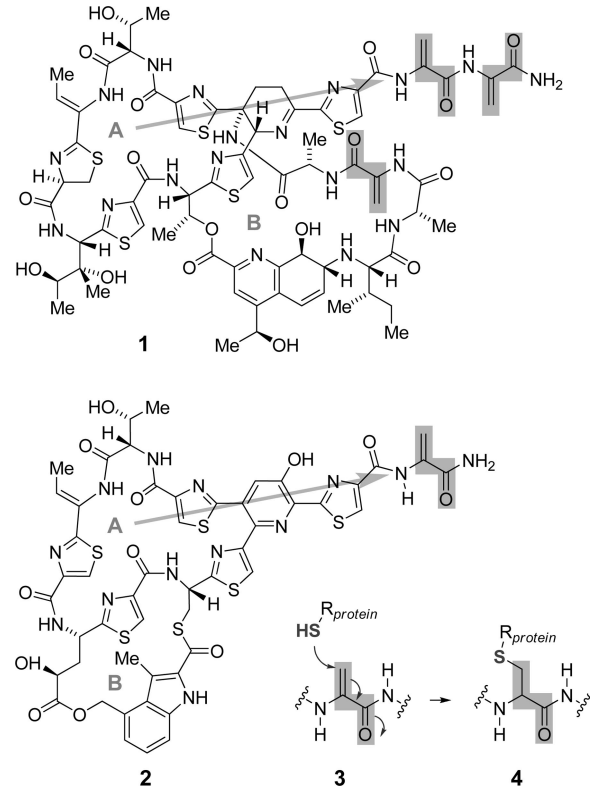


Figure 1. Example for proximity-induced covalent capture (PICC) on formation of a suitable protein/RNA/ligand complex. (A) Ligand binding and (anticipated) structural rearrangement. (B) Covalent reaction of the protein with the ligand (green); then denaturing, isolation, and analysis.

To explore this concept, the thiopeptide binding site was chosen as an important RNA−protein complex, the site of action of the antibiotics[6] Thiostrepton (**1**, Scheme 1) and Nosiheptide (**2**). Detailed studies of **1** have revealed sub-nanomolar affinity for the 50S subunit of the bacterial ribosome.[7] **1** locks the conformation of the flexible ribosomal protein L11 on the ribosomal 23S rRNA[8] and blocks the action of ribosomal GTPases.[9–11] A similar function is generally assigned to **2** due to its highly related structure and activity. However, despite significant investigations by crystallography[12] and NMR,[13,14] the exact location and molecular structure of the thiopeptide binding site remains under debate. An installation of cysteines on the L11 protein should allow studying the PICC of thiopeptides to investigate their binding site in detail. Interestingly, **1** and **2** feature conformationally rigid dehydroalanines which are predisposed to undergo irreversible 1,4-thiol additions (**3** → **4**).[15,16]

The Cys-free L11 protein from *Thermus thermophilus* was selected for further study. Suitable surface positions for the placement of reactive Cys around the thiopeptide binding region were chosen by inspection of X-ray crystal structure data of the ligand-free 23S RNA/L11 complex.[12] Residues conserved in all bacterial L11 proteins were kept constant (Figure 2), as well as

Scheme 1. Molecular Structures of Thiostrepton (**1**) and Nosiheptide (**2**) and the Envisioned Cystein 1,4-Addition Pathway (**3** → **4**)[a]



[a] Reactive Michael acceptor positions are highlighted in gray; light arrows illustrate the vectorial orientation of the dehydroalanine tail with respect to the similar A-rings (compare to Figure 5).

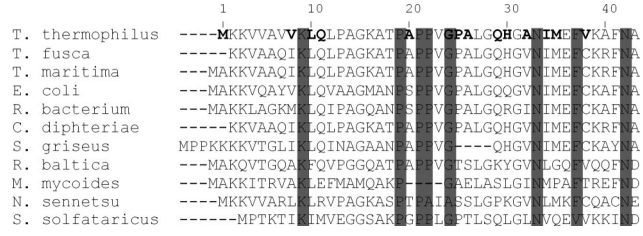


Figure 2. Comparative sequence alignment of the L11 protein N-termini (*T. thermophilus* numbering). Highly conserved residues are highlighted; positions selected for PICC are marked in bold face.

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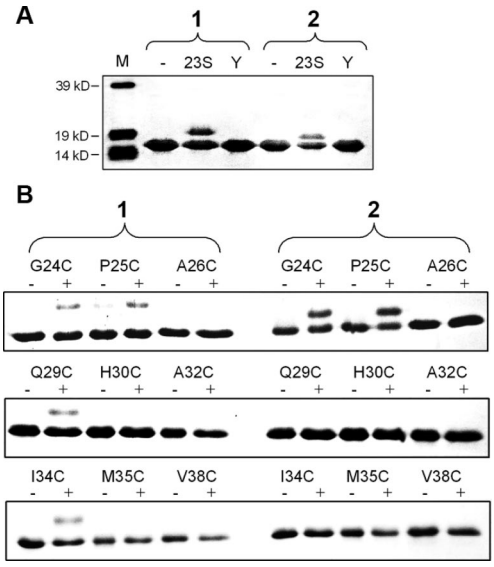

Figure 3. (A) Coomassie-stained denaturing 15% SDS-PAGE gels of Thiostrepton (**1**) and Nosiheptide (**2**) incubated with *T. thermophilus* L11-G24C at 5 μM; (–) no RNA; (+) with 5 μM *E. coli* 23S rRNA (1051–1109); (Y) with 5 μM yeast total RNA extract; (M) molecular weight markers (Invitrogen SeeBlue-2). (B) Positional scan for covalent addition to the L11 mutants at 5 μM of complex resolved by 15% SDS-PAGE; (–) no RNA; (+) with matching *E. coli* 23S rRNA fragment (1051–1109).

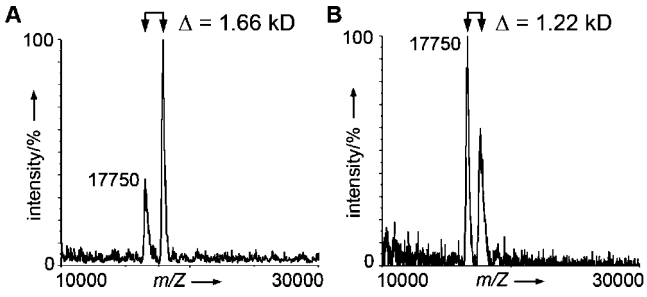

Figure 4. Identification of reactive ligand positions by MALDI-TOF mass spectrometry. (A and B) Full length adducts of L11-G24C to **1** and **2**. MS analyses of tryptic digests can be found in the Supporting Information. M_r(**1**) = 1664.9, M_r(**2**) = 1222.2, M_r(L11-G24C) = 17751.

residues reported[17] to lead to Thiostrepton resistance after mutation. Single Cys exchange mutants were generated for all indicated positions by site-directed mutagenesis (Figure 2).[18] Overexpressed N-terminally His_6-tagged fusion proteins[19] were purified by Ni-NTA chromatography and stored under reducing conditions. All produced protein variants were soluble, contained >95% active sulfhydryl (as verified by titration with Ellman's reagent)[20] and displayed a similar overall fold when compared with the native protein by CD spectroscopy (Supporting Information).

These L11 mutants were initially screened at neutral pH for their reactivity toward Thiostrepton **1** and Nosiheptide **2**. It was found that the 1:1:1 complex of the key[8] 58nt bacterial 23S rRNA fragment, protein, and thiopeptide ligand led to the formation of a new protein product from L11-G24C within hours at biologically relevant concentrations (0.5–5 μM). Typical denaturing conditions (SDS, 8 M urea, heat, PR_3, thiols) were not able to disintegrate the product, indicating covalency. The protein adducts were monitored by denaturing polyacrylamide gel electrophoresis (PAGE), which resolved the covalent modification and the different molecular weights resulting from **1** or **2** (Figure 3).

Neither the absence of RNA nor the presence of a pool of nonmatching RNAs from yeast extract led to appreciable product formation (Figure 3A), which illustrates that the matching 23S RNA sequence is mandatory to promote the covalent attachment. Furthermore, reactions at variable concentrations of RNA clearly showed concentration dependency (Supporting Information) and proved the specific involvement of the matching RNA.[21] Treating the protein with iodoacetamide or reacting Thiostrepton (**1**) with cysteine[22a] prior to the PICC experiment (not shown) suppressed the attachment, indicating that the engineered Cys attached selectively to the thiopeptide's dehydro-Ala by a Michael-type addition. Moreover, independent Michael addition experiments of Cys derivatives to **1** in the absence of the L11/RNA complex indicated that addition occurs preferentially at the dehydro-Ala "tail" (Supporting Information), in line with earlier reports.[22,26]

A panel of L11 protein variants was investigated next (Figures 2, 3B, and Supporting Information). For Thiostrepton (**1**), four reactive positions on the L11 protein could be clearly distinguished: G24C, P25C, Q29C, and I34C. Two weakly reactive mutants were identified in close proximity (A20C, H30C). The PICC reactions with Nosiheptide (**2**) revealed high selectivity. Attachment of **2** to the protein occurred only with the proximal mutants G24C and

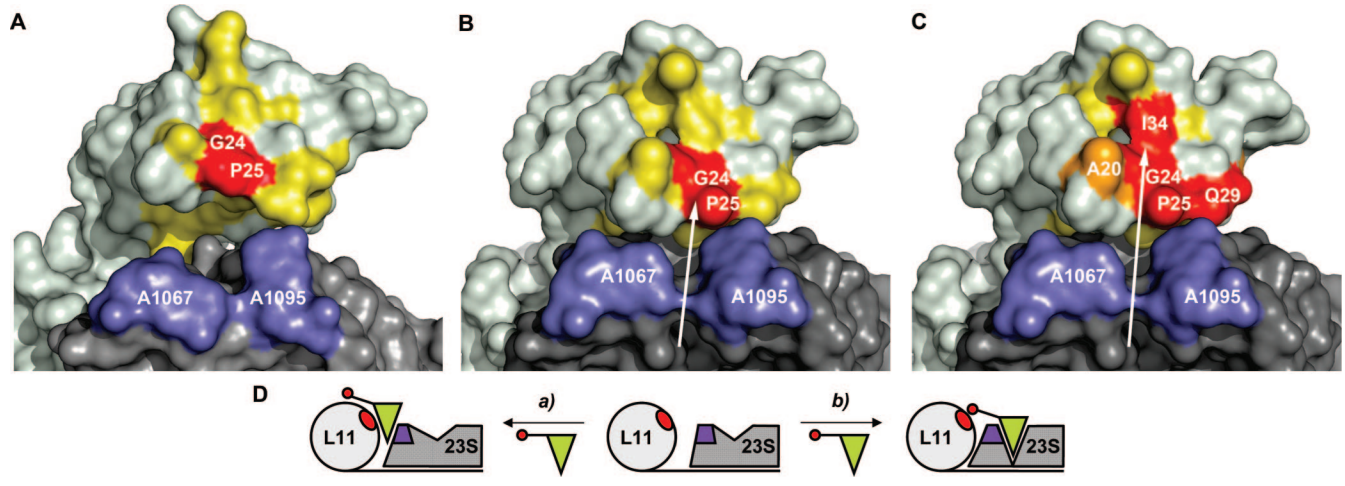

Figure 5. Molecular surface area of L11/rRNA complexes with PICCed positions (color code: red = highly reactive Cys mutant, orange = weakly reactive, yellow = unreactive, gray = not tested, blue = prominently involved nucleobases; light = L11 protein, dark = 23S RNA). The direction of the dehydro-Ala tail is indicated by an arrow (cf. Scheme 1). (A) Reactivity of **2** mapped on PDB 1mms.[12] (B) Reactivity of **2** on PDB 2jq7.[14a] (C) Reactivity of **1** on PDB 2jq7. (D) Cartoon of the proximal[13,14] (*a*) and PICC-derived distal (*b*) binding mode–model; green = thiopeptide ligand with dehydroalanine tail (red dot).

P25C. Some weak background reaction of G24C and P25C was apparent in the case of **1** but not **2**. This could result from a higher tendency of **1** for nonspecific interactions or from some residual specific affinity of the longer Thiostrepton dehydro-Ala tail to this region. Lowering the probing concentration suppressed this effect (Supporting Information). Other nonspecific reactions became prominent only above pH 8.5 or at elevated concentrations. Their uniform appearance ruled out influences of different Cys reactivities due to their potentially different pK_a.[23] It is worth noting that L11 in most bacteria features a Cys at position 38 (Figure 2). However, we did not observe any activity of V38C in our PICC assay, suggesting that the native Cys is not involved in thiopeptide action in vivo.

Examination of crude PICC reaction mixtures and of protein gels by MALDI-TOF mass spectrometry (Figure 4) confirmed the presence of covalent thiopeptide monoadducts (Figure 4A and B). Multiple adducts or degradation products were never observed, corroborating the high selectivity of the molecular recognition event. Proteolytic digestion of PICC reaction products permitted assigning the attachment position to the engineered Cys (Supporting Information). The molecular composition of the covalent attachment could be clearly deduced, thereby signifying that unknown ligands could also be identified by PICC.[4]

The moderate conversions suggest that some structural rearrangement or "slippage" of the ternary complex is necessary for a productive PICC reaction. However, the high spatial selectivity of the PICC experiments indicated a very tight and defined binding site. To gain more insight, the observed reactivity patterns were mapped onto reported structural data of L11/23S RNA complexes (Figure 5).[12,14] Nosiheptide (**2**) with its single Michael acceptor gave a positive PICC only in a very confined area, precisely locating the dehydro-Ala in the ternary complex. In the ligand-free crystal structure, G24/P25 lie near to a cleft (Figure 5A),[12] which was later reported to narrow significantly upon ligand binding (Figure 5B).[14] This cleft is generally too close (8–9 Å) to host a bound **2** which reacts covalently with G24C (distance of the dehydro-Ala to the crucial A-ring >18 Å).[24] On the other hand, the RNA is known to be strongly affected by thiopeptide binding, especially the nucleobases A1067 and A1095.[25] Taken together, these data could suggest that **2** binds mainly to the RNA surface (Figure 5D). An insertion[13,14a] between the L11 protein and the RNA seems less likely.

For Thiostrepton (**1**), three major PICC spots were identified (Figure 5C). Taking this distribution and the structural similarity of **1** and **2** into account, the triangular pattern observed is in full agreement with the longer dehydro-Ala tail of **1** pointing toward the L11 protein from the same putative RNA binding site (arrows in Figure 5), and with the inner dehydro-Ala being positioned at P25. In principle, could this PICC experiment distinguish between two earlier proximal binding models[13,14] which are substantially different.[24a] Interestingly, the PICCs observed here suggest a novel ligand binding mode more distal from the L11 protein on the composite protein−RNA surface, close to the crucial nucleobases A1067 and A1095.[25] Such distal binding to the RNA—not inserted between L11 protein and RNA—would fully agree with the biochemical data available[7–11] and could explain the bioactivity of the Thiostrepton core fragment.[26] Further experimentation is warranted to define this binding site in higher detail.[24b]

In conclusion, we have successfully demonstrated that the affinity reagent principle can be extended to ligands binding to a protein−RNA complex that undergoes major conformational transitions. The positioning of the dehydro-Ala tail of the thiopeptides in the bound complex was clarified, which suggested the rRNA as the major binding region for Thiostrepton (**1**) and Nosiheptide (**2**). The PICC experiment can be easily conducted at micromolar concentrations with no need for additives or catalysts. At present, PICC is conveniently analyzed when the protein reaction products can be resolved by chromatography or electrophoresis. However, it is expected that its scope can be expanded by employing protein digestion or tagging techniques.

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Supporting Information Available: Preparation of protein, RNA, Michael additions, PICC experiments, and MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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