Adducts **7c–e** and **11c–e**: Hydrazone **1** or **5** was solved in excess (about 5 equiv) of ketone (**6c–e**), and the mixture was stirred until consumption of the former (TLC). Unchanged ketone was recovered (75–85%) by bulb-to-bulb distillation, and the residue was purified by flash chromatography.

Compounds **12** and **14** were synthesized from **11** under standard conditions.

Aldehydes **13**: Ozone was bubbled through a solution of **12** (1 mmol) in dry CH_2Cl_2 (5 mL) at $-78\,^{\circ}C$ until appearance of a permanent blue color (5–10 min). Me_2S (5 mmol) was added. The mixture was allowed to warm to room temperature and concentrated, and the residue purified by column chromatography.

Carboxylic acids **15**: Ozonolysis was carried out from **14** as described above, but only 1 mmol of Me_2S was added. To the resulting solution was added *t*BuOH (12 mL) and isobutene (10 mL). After the mixture was cooled to $0\,^{\circ}C$, a solution of $NaClO_2$ (10 mmol) and KH_2PO_4 (9 mmol) in H_2O (12 mL) was added dropwise and the mixture was stirred for 16 h. The solvent was removed, and the residue was treated with 1M NaOH and extracted with Et_2O (2×10 mL). The aqueous layer was acidified to pH 1 (HCl) and extracted with ethyl acetate (10×5 mL). The combined organic layers were then concentrated and purified by flash chromatography.

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[9] Suitable crystals were obtained from light petroleum ether at room temperature. $C_{22}H_{25}F_3N_2O_2$, $M_r = 406.44$, crystal size $0.1 \times 0.4 \times 0.4$ mm, crystal system orthorombic, space group $P2_12_12_1$, $a = 7.4267(10)$, $b = 14.081(2)$, $c = 20.344(3)$ Å, $V = 2127.5(5)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.269$ g cm^{-3}, $1.76 < \Theta < 23.32^{\circ}$, $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å), $T = 296(2)$ K; of 4500 reflections collected, 2830 were independent [$I > 2\sigma(I)$]; 362 parameters, $R = 0.0682$ ($wR = 0.1100$). The crystal was coated with resin epoxy and mounted in a CCD diffractometer. The intensities were corrected for Lorentz and polarization effects. No absorption correction. The structure was solved by Patterson and Fourier methods, and a final mixed refinement was undertaken. Hydrogen atoms were located in a difference synthesis, and their coordinates and isotropic thermal parameters refined, except for H2 whose thermal parameter was fixed. Refinement on F^2 for all reflections. Weighted factors (wR) and all GOFs are based on F^2; conventional R factors are based on F. The configuration of C(5) is based on that known for the auxiliary. Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Center as supplementary publication no. CCDC-102999. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB21EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

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Detection of Specific Noncovalent Zinc Finger Peptide–Oligodeoxynucleotide Complexes by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry**

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All retroviruses, including the human immunodeficiency virus type 1 (HIV-1), encode a gag precursor polyprotein which contains zinc binding domains of the type CCHC (CCHC = Cys-X_2-Cys-X_4-His-X_4-Cys, X = variable amino acid).[1] These zinc-coordinated motifs play an important role in the recognition of viral ribonucleic acid and replication of the virus. The interaction between peptides containing such motifs and single-stranded nucleic acids has been extensively studied,[2, 3] mainly in view of developing antiviral agents for the treatment of the acquired immunodeficiency syndrome (AIDS).[4] The methods used for these investigations are rather time-consuming and expensive. Here we report that matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is suitable for detecting specific noncovalent complexes, and that it is a potential method for rapidly screening antiviral agents. The use of MALDI-MS is now well known for the analysis of high molecular weight biopolymers.[5] However, its ability to detect specific noncovalent complexes is just starting to be explored.[6] Complexes that are stable under physiological conditions in solution may not survive laser desorption and ionization processes. Using carefully designed controls, we were able to establish a correlation between the existence of a specific noncovalent triple complex in solution and in the MALDI mass spectra.

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The peptide selected for the investigation is an 18-residue peptide of the type CCHC with the amino acid sequence acVK**C**FN**C**GKEG**H**IARN**C**RA-OH. This sequence corresponds to the first zinc finger domain from the gag protein p55 of HIV-1, called p55F1. The peptide has no defined secondary structure in the absence of metal ions. Metal-ion binding induces peptide folding, which is required for the interaction with nucleic acids.[2, 7] Peptides of the type CCHC bind Zn^{2+} very tightly, whereas complexes with other transition metal ions are weaker.[8] In the Zn – p55F1 complex, Zn^{2+} is tetrahedrally coordinated by the three cysteine and the histidine residues.[9] A preliminary MALDI-MS study of the complexation between p55F1 and various metal ions was performed in our laboratory.[10] We found that the Zn – p55F1 complex detected is specific, and that the complex formation observed qualitatively correlates with that in solution (see also reference [11]).

We now investigated the triple complex Zn – p55F1 – d(TTGTT) with MALDI-MS, because it is known from solution experiments that d(TTGTT) binds to Zn – p55F1.[3] The oligodeoxynucleotide binds within a hydrophobic cleft on the peptide surface. For reasons of sample stability, it is common practice to use single-stranded oligodeoxynucleotides as structural probes for ribonucleic acid binding CCHC zinc fingers.[3]

All MALDI mass spectra were taken in the positive-ion mode on a linear time-of-flight instrument that has been described in detail before.[11] Figure 1 A shows the MALDI mass spectrum of a mixture of d(TTGTT) and p55F1. The signals for the protonated molecular ions of p55F1 ($[P+H]^+$) and d(TTGTT) ($[O+H]^+$) are observed besides weak peaks for an oligodeoxynucleotide fragment (*m*/*z* 1399) and a nonspecific adduct of both components. The latter is probably a result of electrostatic interactions. Upon addition of Zn^{2+}, the signal for the specific triple complex of p55F1, Zn^{2+}, and d(TTGTT) is observed (Figure 1 B, *m*/*z* 3598). Less intense signals for p55F1 – d(TTGTT) adducts without Zn^{2+} and with two Zn^{2+} ions are also observed. All signals correspond to singly charged species. The excess charge of Zn^{2+} is compensated by deprotonation of the peptide or the oligodeoxynucleotide, as confirmed by high-resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometry. Adduct peaks at higher mass were of negligible intensity.

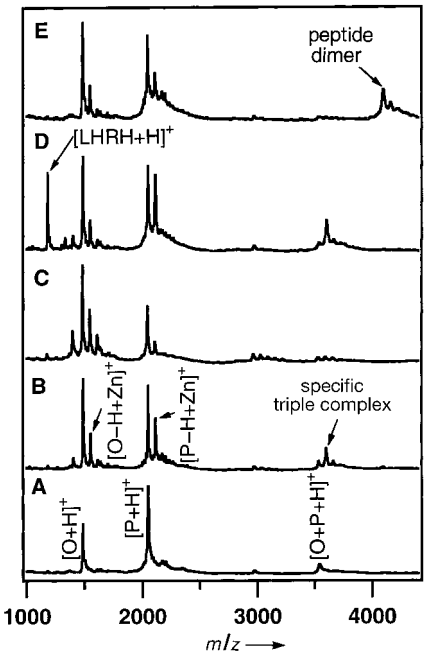

Figure 1. MALDI mass spectra (AMNP matrix) of p55F1 and d(TTGTT) in a 33:1 molar ratio A) without addition of Zn^{2+} at pH 6.5 – 7, B) with addition of Zn^{2+} (molar ratio Zn^{2+}:p55F1 = 5:1) at pH 6.5 – 7, C) same as (B) at pH 5 – 5.5, D) same as (B) with addition of LHRH at pH 7, E) same as (B) with addition of Cu^{2+} in the same molar amount as Zn^{2+}. The metal ion adducts to p55F1 (*m*/*z* 2113) and to its dimer (*m*/*z* 4262) stem from a nonspecific gas-phase reaction.

The signals of both the triple complex and the Zn – p55F1 complex are less intense than those of the individual components. The reasons in the case of Zn – p55F1 have already been discussed.[10] Similar arguments can also be made for the triple complex: Variations in desorption, ionization, and detection efficiencies among the triple complex, the Zn – p55F1 complex, the peptide (present in excess), and d(TTGTT) as well as partial peptide oxidation or partial dissociation of the complex ions upon crystallization or during the MALDI process may all contribute to the triple complex signal being less intense than that of the components. From this, it is clear that absolute abundances of the species in solution (the complex and its components) and peak intensities in the MALDI mass spectra cannot be directly compared.[10]

Furthermore, conclusions concerning complex specificity cannot be drawn purely on signal intensities. Carefully designed control experiments have to be performed, such as the variation of the pH or competition experiments. Such controls have already been done to prove the specificity of Zn – p55F1.[10] Similar controls for Zn – p55F1 – d(TTGTT) are described below.

The investigation of Zn – p55F1 with circular dichroism (CD) spectroscopy in solution showed that this complex is only stable above pH 6.[10] This is what we also expect for the triple complex, since the oligodeoxynucleotide only binds to the zinc-complexed p55F1. If MALDI mass spectra reflect solution-phase behavior, then decreasing the pH to a value below 6 should lead to a significant decrease in the MALDI signal of the specific triple complex. This is exactly what is observed experimentally (Figure 1 C): Besides a strong decrease of the Zn – p55F1 signal compared to that of p55F1, the triple complex signal is absent. Instead, weak signals of a nonspecific distribution of Zn^{2+} adducts to p55F1-d(TTGTT) and to the oligonucleotide dimer are observed. We assume that the negatively charged phosphodiester backbone of the oligonucleotide forms nonspecific adducts with the overall positively charged p55F1 and with Zn^{2+} ions. This assumption fits well with the observation of multiple Zn^{2+} adducts to the oligonucleotide in the mass spectrum in Figure 1 C.

To confirm whether the triple complex observed in Figure 1 B is indeed specific, another peptide (luteinizing hormone releasing hormone, LHRH, *m*/*z* 1182) was added to the sample (Figure 1 D). The peptide LHRH contains a histidine residue in its sequence and thus potentially binds to

Zn^{2+} ions. It also contains arginine with a positively charged side chain, which may bind through electrostatic forces to the phosphodiester groups of the oligodeoxynucleotide backbone. Both interactions would be nonspecific. The spectrum shows that neither a Zn^{2+} adduct to this peptide (expected at m/z 1246) nor a triple complex with Zn^{2+} and d(TTGTT) (expected at m/z 2730) is detected. However, the corresponding complex signals of p55F1 are as intense as in the absence of LHRH (Figure 1 B). They are even more intense because of a slightly higher pH value. This experiment supports the interpretation that the complex formation of Zn^{2+} with p55F1 and d(TTGTT) observed with MALDI-MS is specific.

The study of p55F1 with different metal ions showed that an excess of Cu^{2+} with respect to p55F1 oxidizes the peptide's thiol functionalities to an intramolecular disulfide bond with simultaneous reduction of Cu^{2+} to Cu^{+}.[10] The Cu^{2+} ion mimics the action of an antiviral agent in the sense that it chemically modifies the cysteine residues. Modified p55F1 forms complexes neither with Zn^{2+} nor with oligodeoxynucleotides. This is also reflected in the MALDI mass spectrum: If Cu^{2+} is added to the sample, the cysteine residues are oxidized and the signal of the triple complex disappears (Figure 1 E). A strong signal for the p55F1 dimer is detected instead, because peptide oxidation can lead to intermolecular disulfide bonds involving the third thiol group.

Besides d(TTGTT), the complexation of Zn – p55F1 with other sequences such as d(TTATT) and d(ACGCC) has also been investigated. Our MALDI experiments with d(TTATT) instead of d(TTGTT) support the findings of Lam et al.[2] and Gorelick et al.,[12] who report that Zn – p55F1 is not sequence-specific in solution. We found that the complex of d(TTATT) with Zn-p55F1 gave signals of about the same intensity as with d(TTGTT). MALDI experiments with oligodeoxynucleotide sequences lacking thymidine are, unfortunately, not very informative. If d(ACGCC) was used as the binding partner hardly any MALDI signal could be seen at all, although the corresponding triple complex should be stable in solution.[3] It is common knowledge that oligodeoxynucleotides that do not contain thymidines are much less stable, more prone to fragmentation, and thus much more difficult to detect under the same experimental MALDI conditions than those containing mostly thymidine residues.[13]

In summary, the present MALDI study demonstrates that specific, noncovalent complexes between Zn^{2+}, the zinc finger peptide p55F1, and different oligodeoxynucleotides can be detected with MALDI-MS. Moreover, this methods allows effects of pH and of chemical modifications of the zinc finger peptide on the formation of the triple complex to be studied. Therefore, MALDI-MS is a potential method for rapidly sceening antiviral agents. More generally, this work not only demonstrates the potential of MALDI-MS to rapidly and directly analyze noncovalent complexes, but also shows that the mass spectral data can, under carefully chosen conditions, reflect solution-phase chemistry.

Experimental Section

P55F1 (m/z 2049) was synthesized on TentaGel S resin from Fmoc-protected amino acid derivatives (Fmoc = (9*H*-fluoren-9-ylmethoxy)carbonyl), and purified on a reverse-phase high-performance liquid chromatography C18 column. As the thiol groups of the three cysteine residues in the CCHC motif are sensitive to oxidation, p55F1 was lyophilized, stored under an argon or nitrogen atmosphere, and handled rapidly during the experiments to minimize oxidation. The oligodeoxynucleotides d(TTGTT) (m/z 1484), d(TTATT), and d(ACGCC) were purchased in the desalted form from Microsynth, Balgach, Switzerland. Further desalination by the Millipore "drop dialysis method"[10] yielded MALDI mass spectra showing only the protonated molecular ion peak of the oligodeoxynucleotide and hardly any alkali ion adducts. 2-Amino-4-methyl-5-nitropyridine (AMNP) was used as a MALDI matrix for the analysis of the Zn – p55F1 – oligodeoxynucleotide complexes. This nonacidic matrix is optimized for MALDI-MS of oligodeoxynucleotides,[14] but allowed the simultaneous detection of both p55F1 and oligodeoxynucleotide at the physiological pH of the sample solution. For sample preparation, the aqueous peptide solution (10 μL, 5×10^{-4} M) was mixed with an aqueous solution of $ZnCl_2$ (2.5 μL, 10^{-2} M) and an aqueous oligodeoxynucleotide solution (2.5 μL, 6.1×10^{-5} M). The concentration of $ZnCl_2$ is unusually high for MALDI experiments, but was found to work well. The pH value was adjusted to 6.5 – 7 by addition of NH_4HCO_3. An aliquot (3.5 μL) of this mixture was mixed with a saturated, aqueous AMNP solution (10 μL). A portion (2 – 4 μL) of the resulting solution was deposited on the probe tip and dried in a cold stream of air.

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