Structural probing and damage selection of citrulline- and arginine-specific RNA aptamers identify base positions required for binding

Petra Burgstaller, Michel Kochoyan[1] and Michael Famulok*

Institut für Biochemie der LMU München - Genzentrum, Würmtalstraße 221, 81375 München, Germany and [1]CNRS-URA 1254, Groupe de Biophysique, Ecole Polytechnique, Route de Saclay, 91128 Paliseau, France

ABSTRACT

In a recent study, an RNA aptamer for the specific recognition of the amino acid L-arginine was evolved from an *in vitro* selected L-citrulline binding parent sequence [M. Famulok (1994) *J. Am. Chem. Soc.* 116, 1698–1706]. We have now carried out a structural analysis of these aptamers by using chemical modification experiments. Footprinting experiments and a damage selection approach were performed to identify those positions protected from modification in the presence of the amino acids and modifications that interfere with the binding of the ligand. It is shown that of the two bulged regions present in both aptamers one can be modified without loss of binding activity whereas in the other bulge nearly every position is shown to be involved in the recognition of the ligands. This might be indicative for non-canonical base pairing to occur within the non-Watson–Crick paired regions which might be stabilized by the complexed amino acid. Binding to the cognate amino acid significantly enhances the conformational stability of the RNA. We also tested the sensitivity of both aptamers towards lead (II) ion induced cleavage and identified a hypersensitive cleavage site within the invariant bulged region. Lead cleavage is inhibited by the complexed amino acid, indicating a conformational change of the aptamer upon ligand binding. NMR titration data obtained with both aptamers and their cognate ligands confirm the proposed conformational changes and indicate the formation of a 1:1 complex of RNA:amino acid.

INTRODUCTION

Since the development of the *in vitro* selection or SELEX technique in 1990, more than a dozen RNA or DNA aptamers which specifically recognize molecules of low molecular weight have been reported. Examples range from natural molecules such as amino acids (1–4), biological cofactors (5–9), alkaloids (10) and aminoglycoside antibiotics (11–13) to abiotic organic dyes (14,15) and transition state analogues (16,17). Furthermore, nucleic acid aptamers which bind to and sometimes inhibit the

activity of a great variety of proteins have been isolated [for reviews see e.g. (18–21)]. Among all these different sequences to date only one exists for which a detailed structural characterization, based on comprehensive NMR analyses, has been carried out. This sequence, a 15mer ssDNA inhibits human thrombin (22) by forming a G-quartet structure which binds into the exo-site of the blood-clotting factor (23–25). Consequently, the structural analysis has permitted understanding in great detail of how the aptamer binds and inhibits the function of thrombin. For some RNA aptamers chemical modification analyses have been carried out to confirm proposed secondary structures and to test qualitatively whether conformational changes are induced upon ligand binding. Comprehensive analyses of aptamer structures by chemical probing have been performed in a few cases (26).

Here we used chemical probing with the specific structure probes DMS, kethoxal and CMCT to examine the secondary structure of a citrulline binding RNA aptamer and its related arginine binding triple mutant (27). These two aptamers have been obtained in a recent study in which the SELEX technique was applied to isolate RNA aptamers for the amino acid L-citrulline and then to evolve this binding motif for the recognition of the structurally related amino acid L-arginine. The proposed secondary structure of these RNAs, a common 44mer minimal motif, was solely built on the basis of covariations among the selected sequences. The secondary structure consisted of two stem regions variable in base composition and length which flank two internal bulge regions including a small two base-pair stem (Fig. 1). This motif bound L-citrulline with a K_d of 68 μM. The triple mutant bound to L-arginine with a K_d of 60 μM and had almost completely lost its affinity for L-citrulline ($K_d = 7.19$ mM).

To analyze the proposed secondary structure we carried out footprinting experiments in the absence and presence of the amino acids. Furthermore, to identify those base positions important for binding of the ligands, a modification–selection approach (damage selection) was performed. In addition, in order to gain information about potential metal binding sites within these aptamers, their sensitivity towards lead-ion induced cleavage was tested. Finally, we used an NMR titration analysis to gain information about conformational changes and to see whether we could draw conclusions about the stoicheometry of complexation of the amino acid and the RNA aptamer.

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* To whom correspondence should be addressed

Table 1. RNAs used for modification and NMR experiments, and primers used for PCR amplification

Name of RNA	Sequence
44Cit11	5'GACGAGAAGGAGUGCUGGUUCUACUAGCGGUUAGGUCACUCGUC3'
44Arg11	5'GACGAGAAGGAGCGCUGGUUCUACUAGCAGGUAGGUCACUCGUC3'
64Cit11	5'GACGAGAAGGAGUGCUGGUUCUACUAGCGGUUAGGUCACUCGUCAUACCACUAUAUUAGAGGUA3'
64Arg11	5'GACGAGAAGGAGCGCUGGUUCUACUAGCAGGUAGGUCACUCGUCAUACCACUAUAUUAGAGGUA3'
Cit.NMR	5'GACGGUUAGGUCGCACGAAAGUGAAGGAGUGUC3'
Arg.NMR	5'GACAGGUAGGUCGCACGAAAGUGAAGGAGCGUC3'
20PB.1 (primer)	5'TACCTCTAATATAGTGGTAT3'

MATERIALS AND METHODS

Chemicals and enzymes

Dimethylsulfate (DMS) was purchased from Aldrich, kethoxal from ICN and 1-cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide metho-*p*-toluenesulphonate (CMCT) from Sigma.

[γ-^{32}P]ATP (3 Ci/µmol) was from Amersham. T7 RNA polymerase was purified from the overproducing strain BL21/pAR1219, following the purification protocol provided by F.W. Studier (28). AMV reverse transcriptase and DNase I (RNase free) were from Boehringer Mannheim, T4 polynucleotide kinase from Biolabs. Arginine and citrulline were purchased from Sigma. Ligand derivatized agarose was synthesized from epoxy-activated Sepharose 6B (Pharmacia) as described previously (4).

Preparation of DNA and RNA

The RNAs used for this study are listed in Table 1.

RNAs were obtained by *in vitro* transcription from DNA templates containing a T7 promotor (29). DNA templates were generated by PCR amplification of synthetic oligonucleotides. PCR reactions were performed in PCR-buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 0.001% gelatine, 1.5 mM MgCl$_2$, 0.3% Tween 20, 0.2 mM dNTPs) in the presence of 3 µM primer. For NMR analyses, RNA was generated by T7-transcription of a synthetic (−)-DNA-oligonucleotide hybridized to a (+)-T7-promotor oligonucleotide. Primers and synthetic oligonucleotides used in PCR amplification reactions were synthesized on a Millipore Expedite oligonucleotide synthesizer using standard phosphoramidite chemistry.

RNAs for lead cleavage experiments were radiolabelled with ^{32}P at the 5'-end. All RNAs were purified by preparative gel electrophoresis on denaturing polyacrylamide–8.3 M urea gels. RNAs for modification experiments were aditionally purified by affinity chromatography on amino-acid derivatized agarose columns. The RNA was applied to epoxy activated Sepharose 6B from Pharmacia derivatized with the respective amino acid (~20 mM). After washing the agarose column with five column volumes of selection buffer (250 mM NaCl, 50 mM Tris pH 7.6, 5 mM MgCl$_2$), binding molecules were eluted with a 60 mM solution of ligand in selection buffer. The eluted RNA was ethanol precipitated in the presence of 10 µg tRNA, dissolved in water, passed over a G-50 Sepharose column, reprecipitated and redissolved in water.
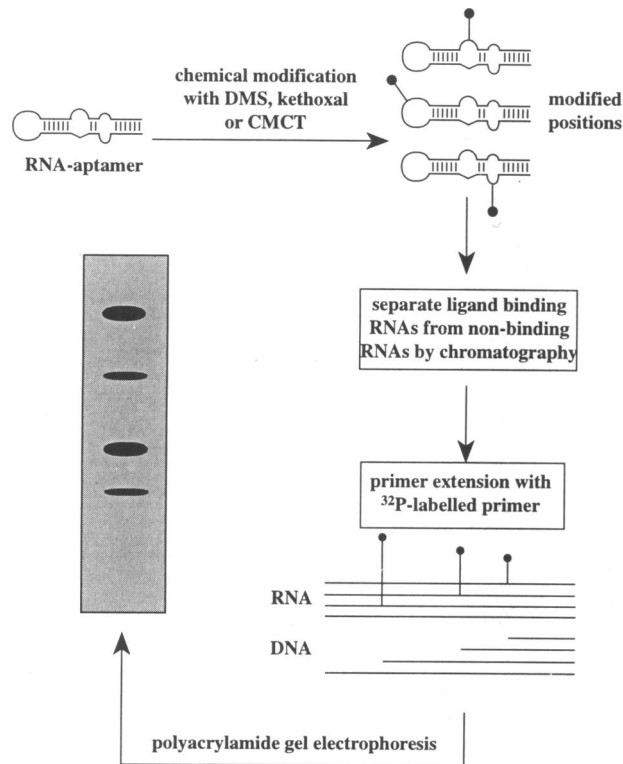
Chemical modification and damage selection

After 3 min of denaturation at 95°C, 1–5 pmol RNA 64Cit11 or 64Arg11 was renatured in the absence or presence of 5 mM of
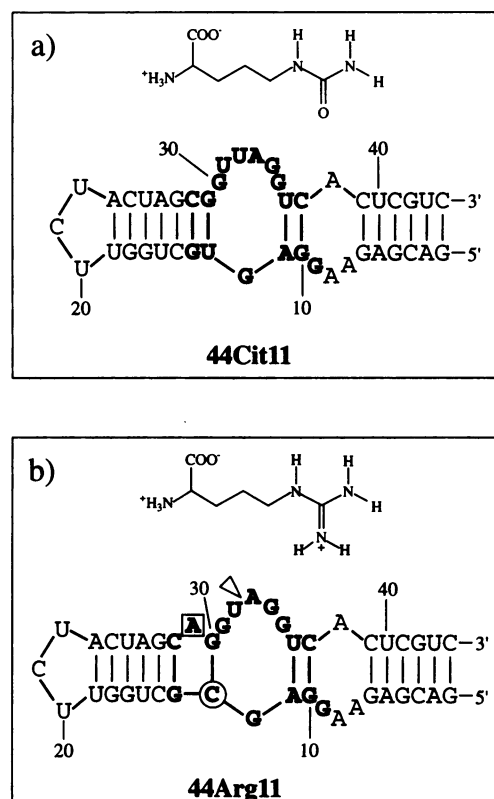
Figure 1. Chemical structures of ligands and secondary structures of RNA aptamers. (a) L–citrulline and sequence and secondary structure of 44Cit11, the citrulline specific aptamer. (b) L–arginine and sequence and secondary structure of 44Arg11, the arginine specific triple mutant of 44Cit11. The proposed secondary structures were based on covariations among different selected clones. The 6- and 10mer consensus sequences are shown in bold. The box, circle and triangle indicate the mutations in the arginine aptamer relative to the citrulline aptamer.

amino acid L-citrulline or L-arginine in 250 mM NaCl, 50 mM sodium cacodylate pH 7.4 and 5 mM MgCl$_2$ for 10 min at room temperature. For probing with CMCT 50 mM potassium borate pH 8.0 instead of sodium cacodylate was used. Chemical modification (27,30) was performed by addition of 1 µl DMS (1:5 dilution in 96% ethanol), 1 µl kethoxal (1:10 dilution in H$_2$O, stock solution: 37 mg/ml) or 12.5 µl CMCT (42 mg/ml in H$_2$O) to a final reaction volume of 50 µl, followed by incubation at 25°C for 20 min. After precipitation in the presence of 10 µg tRNA, samples were dissolved in H$_2$O for DMS and CMCT
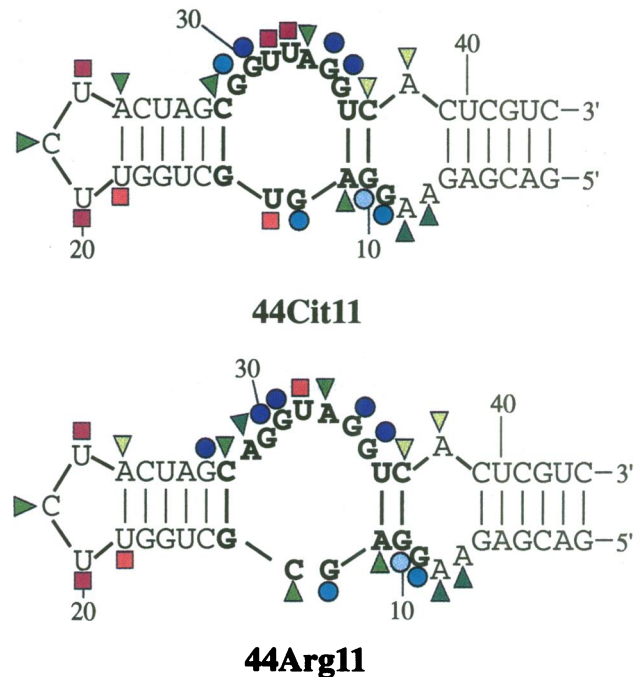
Figure 2. Schematic for the damage selection approach. The RNA is applied to chemical modification using specific structure probes like DMS, kethoxal or CMCT. The modifications are introduced at a level of less than one modification per molecule. Molecules of the modified population that are still able to bind to their cognate ligand are separated from non-binding sequences by affinity chromatography. After primer extension of the isolated RNAs, the cDNAs are analyzed on denaturing polyacrylamide gels.

44Cit11

44Arg11

Figure 3. Summary of the reactivity of nucleotides in the free RNA to the chemical probes DMS, kethoxal and CMCT.

modified samples or 25 mM potassium borate pH 7.0 for kethoxal modified samples.

For damage selection experiments, modified RNA was renatured in selection buffer (see above; for kethoxal modified RNAs, 25 mM K-borate pH 7.0 was added) and applied on ligand-derivatized agarose (~20 mM). After washing with five column volumes of buffer, the remaining bound molecules were eluted with a 60 mM solution of amino acid in selection buffer. The eluted RNA was precipitated and dissolved as described above.

For probing of the N7 positions of guanosine, an aniline-induced strand scission was performed. After precipitation, the modified RNA was dissolved in 1 M Tris–HCl pH 8.2 and incubated in 0.1 M $NaBH_4$ for 20 min on ice in the dark. The RNA precipitated again and the pellet was incubated in 10 µl of a solution (pH 4.5) containing 8.8% aniline, 27.2% acetic acid, and 64% water for 15 min at 60°C in the dark. The solution was extracted with phenol, washed with chloroform, precipitated, and redissolved in H_2O. Detection of modified positions by primer extension and polyacrylamide gel electrophoresis was performed as described previously (30) using 5′-^{32}P end-labeled primer 20.PB1.

Lead cleavage experiments

5′-^{32}P-end-labeled RNA 44Cit11 or 44Arg11 was renatured in selection buffer in the presence of 5 µg yeast tRNA as described above. Pb(OAc)$_2$ was added to 0.2, 0.5 or 1 mM and the solution was incubated at 25°C for 20 min. The reaction was stopped by addition of EDTA to 2 mM. The RNA was precipitated, redissolved in H_2O and analyzed on polyacrylamide–8.3 M urea gels.

RESULTS AND DISCUSSION

Experimental strategy

For the chemical modification experiments, the RNAs 64Cit11 and 64Arg11 were synthesized identical to the 44mer minimal binding motifs shown in Figure 1, but with an additional single stranded region of 20 nucleotides at the 3′-end to allow the annealing of a complementary primer in the primer extension analysis. To minimize the portion of molecules folded in a non-binding conformational state after renaturation, the RNAs were applied to affinity chromatography on ligand-derivatized agarose. More than 90% of the RNAs purified by this method bound specifically when loaded onto a ligand derivatized agarose column for a second time compared with 50–55% of RNAs not treated that way (data not shown). The RNAs were incubated in the absence
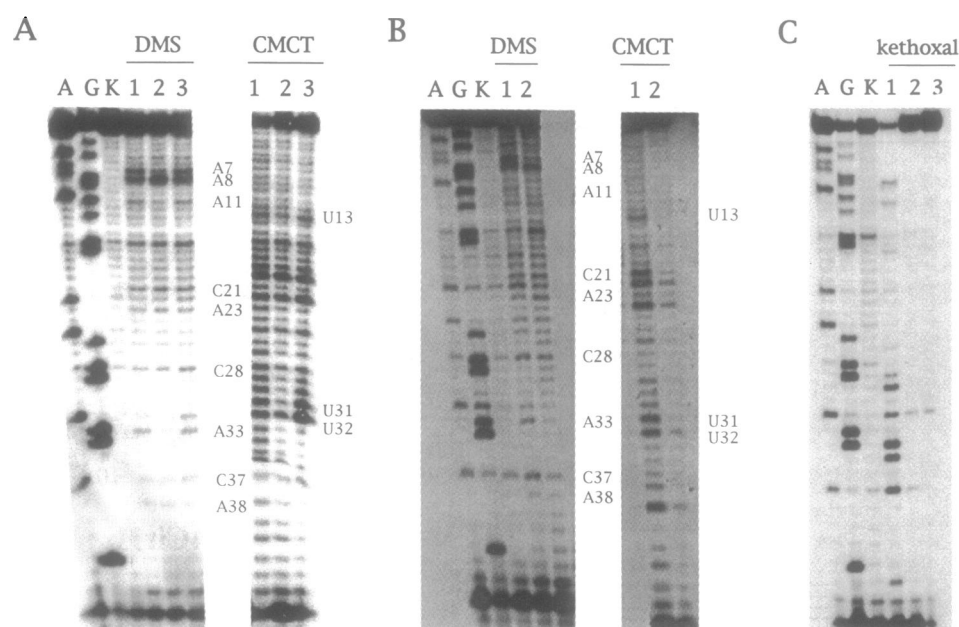
Figure 4. Footprinting and damage selection experiments of 64Cit11 with DMS, CMCT and kethoxal. A and G indicate the dideoxy sequencing lanes, K the primer extension of unmodified RNA. (**A**) The modification of 64Cit11 with DMS and CMCT. Lane 1, free RNA; lane 2, RNA in the presence of 5 mM cognate amino acid; lane 3, RNA in the presence of 5 mM non-cognate amino acid. (**B**) The damage selection data of 64Cit11 modified by DMS and CMCT. Lane 1, modification of free RNA; lane 2, RNAs which retained binding activity separated from non-binding RNAs by affinity chromatography. (**C**) The footprinting and damage selection data of 64Cit11 with kethoxal. Lane 1, modification of free RNA; lane 2, RNAs which retained binding activity separated from non-binding RNAs by affinity chromatography; lane 3, RNA modified in the presence of 5 mM of cognate ligand.

or presence of cognate and non-cognate amino acid under the same buffer conditions applied during the selection procedure. DMS, kethoxal and CMCT were subsequently added to specifically modify bases at accessible positions as described previously (30). In addition to the footprinting experiments in the presence of ligand, a damage selection approach was undertaken to localize the nucleotides important for the interaction with the amino acids (Fig. 2).

The aptamers were modified at approximately one modification per molecule, and RNAs that were able to bind to the appropriate amino acid were separated from non-binding sequences by affinity chromatography on the cognate amino acid derivatized agarose. Those molecules which retain binding activity are expected to contain base modifications only at positions where modification is tolerated. Modifications not tolerated in the selected portion in comparison to the total population reflect those sites needed for the recognition of the amino acid—either because the sites are neccessary to form tertiary interactions upon amino acid binding, or because they directly contact the ligand.

Secondary structure probing of the free RNA

To examine the secondary structure of the L-citrulline and L-arginine binding aptamers, modification at classical Watson–Crick base pairing positions with DMS, kethoxal and CMCT were carried out. Figure 3 summarizes the modification pattern of both aptamers shown in Figure 4 (citrulline aptamer) and Figure 5 (arginine aptamer).

These results confirm the secondary structure suggested for the minimal binding motifs by sequence comparison of the selected pools with the only limitation that the initially proposed base pair G29-U13 (Cit) and G30-C13 (Arg) are unlikely to form. In the

internal loop structure of the citrulline aptamer consisting of the bulged G12 and G30-U31-U32-A33-G34-G35, the region which was invariant among different selected clones, all bases show medium to high degrees of modification. This also applies to the internal bulge of the arginine aptamer (G12 and G31-U32-A33-G34-G35). Likewise, A29 of the arginine binding motif and A7, A8 and G9 in both aptamers are accessible to modification which corresponds to the proposed secondary structure. Furthermore, the observation that G10, A11 and U36, C37 show no or only weak modification is in accordance to the suggested secondary structure models. In both aptamers, A38 shows only weak modification, which argues against our initial suggestion that this position is bulged (4). This base position is interesting because it is not conserved among different isolated sequences. In most clones, A38 can be substituted by a G38 but there are also several clones which have a second bulged base (a purine in every case) at this position while retaining binding activity. A construct in which the bulged R38 is deleted so that a continuous stem can be formed in the upper strand, however, loses its binding activity. Taken together, these results indicate that this base participates in a tertiary interaction involving N-1.

C13 in the arginine aptamer is moderately accessible to modification and thus, it is not clear that base pair C13-G30 is actually formed. This proposal is supported by the strong modification of G30 with kethoxal. When compared to the analogous proposed base pair U13-G29 in the citrulline aptamer, U13 is also moderately modified by CMCT. Note that in contrast to the G30(Arg), however, the G29(Cit) is clearly less accessible to modification with kethoxal. These observations suggest that base pairing C13-G30 (Arg) and U13-G29 (Cit), respectively, does not occur as depicted in Figure 1. However, we are aware that bases which flank unpaired regions in an RNA are sometimes
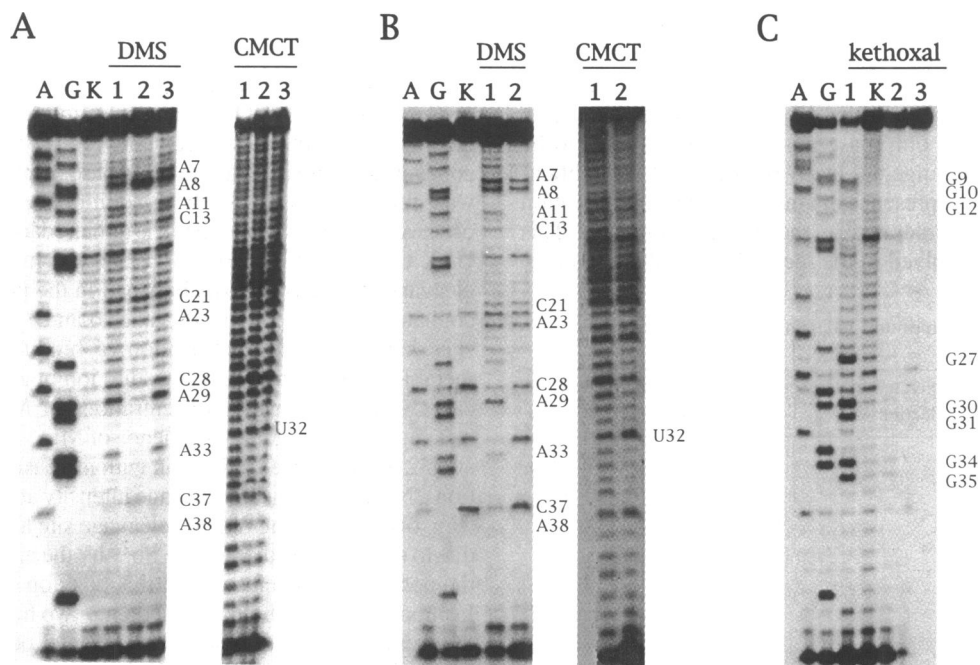
Figure 5. Footprinting and damage selection experiments of 64Arg11 with DMS, CMCT and kethoxal. A and G indicate the dideoxy sequencing lanes, K the primer extension of unmodified RNA. (**A**) The modification of 64Arg11 with DMS and CMCT. Lane 1, free RNA; lane 2, RNA in the presence of 5 mM cognate amino acid; lane 3, RNA in the presence of 5 mM non-cognate amino acid. (**B**) The damage selection data of 64Arg11 modified with DMS and CMCT. Lane 1, modification of free RNA; lane 2, RNAs which retained binding activity separated from non-binding RNAs by affinity chromatography. (**C**) The footprinting and damage selection data of 64Arg11 with kethoxal. Lane 1, modification of free RNA; lane 2, RNAs which retained binding activity separated from non-binding RNAs by affinity chromatography; lane 3, RNA modified in the presence of 5 mM of cognate ligand.

accessible to modification to some extent, although they are actually part of a stem and base pair in a Watson–Crick sense.

G27 in the arginine aptamer which is supposed to form a base pair with C15 shows a strong modification, whereas G27 in the citrulline aptamer is not accessible to modification. At present, we cannot give an explanation for this difference in the modification pattern of the two aptamers.

Secondary structure probing in the presence of the ligand and damage selection

Figures 4 and 5 show the data of the footprinting experiments in presence of 5 mM amino acid and the results of the damage selection assay which are summarized in Table 2.

In contrast to the free aptamers every single position within the invariant internal bulge is inaccessible to modification in the presence of the cognate amino acid. The only exception is U32. At this position no difference in the degree of modification with or without amino acid can be observed. To test whether the observed differences in the modification pattern are due to the presence of the cognate amino acid we also carried out footprinting experiments with the non-cognate amino acid (i.e. arginine for the citrulline aptamer and *vice versa*). We found that the modification pattern obtained did not differ from the pattern obtained with the free RNAs. The damage selection experiments confirm the footprinting data, since modification at the aforementioned bases interferes with binding to the ligand. Interestingly, U13 of the citrulline aptamer and C13 of the arginine aptamer, respectively, show some protection in the presence of amino acid. Modification at this position, however, is clearly not tolerated in the damage selection

showing that this position is important for the recognition of the ligand. This difference between the free and the complexed aptamers is even more obviously manifested at the base position G30 (Arg), the potential partner for a Watson–Crick base pair with C13. In the citrulline aptamer this tendency can also clearly be observed at the related G29. However, since the modification at G29 (Cit) is weaker than that of G30 (Arg) in the free aptamers, the effect is less obvious than in the arginine binder. No modification can be detected at these Gs both in the damage selection and in the footprints. When compared with the clear modification of the free RNAs at positions G29/U13 (Cit) and G30/C13 (Arg), the results obtained in the damage selection and in the footprinting analysis show that the complexed amino acid forces the aptamers either to form a Watson–Crick base pair or, more likely, to stabilize tertiary interactions at these positions. It is also possible that one or both of these bases directly contact the ligand. Probing of the N7 position of the G-bases with DMS/aniline revealed that the N7 of all Gs within the internal loops except G31 (Arg), and to a weaker extent G35 in both aptamers, are accessible to modification in the absence and presence of the cognate ligand (data not shown).

While A7 and A8 are both modified in the free RNAs the modification pattern in the presence of the ligand was different: modification at A8 was strongly enhanced whereas A7 showed slightly weaker accessibility to DMS probing. When the same experiment was performed in the presence of the non-cognate amino acid, the same modification pattern as with the free aptamers was obtained, showing that the observed differences are indeed due to amino acid complexation. The damage selection shows that modification of A7 and A8 with DMS does not

interfere with binding and hence confirms the footprinting results. The observed hypersensitivity to DMS probing of A8 in the presence of cognate amino acid in both aptamers is a clear indication for a conformational change of the RNA structure upon ligand binding. G9, the only base position that is shown to be conserved in the small bulge, is clearly protected from modification with kethoxal in the presence of amino acid, and modification at this position is not tolerated in the damage selection. This indicates that G9 is involved in the recognition of the ligands.

Table 2. Summary of the footprinting and damage selection data

Base position	Tolerated in damage selection	Modified in presence of amino acid
64Cit 11		
A7	+	+
A8	+	++
G9	–	–
G10	–	–
G12	–	–
U13	–	–
C28	+	+
G29	–	–
G30	–	–
U31	–	–
U32	+	+
A33	–	–
G34	–	–
G35	–	–
A38	+	+
64 Arg11		
A7	+	+
A8	+	++
G9	–	–
G10	–	–
G12	–	–
C13	–	–
C28	+	+
A29	–	–
G30	–	–
G31	–	–
U32	+	+
A33	–	–
G34	–	–
G35	–	–
A38	+	+

(+) indicates that modification is tolerated in the damage selection or that the residue is modified in the presence of 5 mM ligand, (–) indicates that modification is not tolerated in the damage selection or that the residue is protected from modification in presence of 5 mM ligand.

Lead cleavage experiments

Pb(II)-induced RNA hydrolysis is a useful tool to probe the tertiary structure of a variety of RNAs. This approach has been successfully applied to structural studies of several tRNAs (31–37), mouse U1 small nuclear RNA (38), *Escherichia coli* 16S RNA (39), RNase P (40,41), and 5S rRNA (42). To gain further information about the structural features of the amino acid binding aptamers, we examined whether specific lead cleavage sites could be found in the minimal binding motifs. Therefore, the

5'-end labeled 44mers 44Cit11 and 44Arg11 were exposed to lead (II) acetate and analyzed by polyacrylamide gel electrophoresis. Lead-ion induced cleavage is found in both aptamers at the conserved base U32 at Pb^{2+} concentrations of ~500 µM (Fig. 6A, lane 3; data not shown for the arginine aptamer). At this concentration, no cleavage at other base positions in the aptamers was found. At 1 mM Pb^{2+}, additional cleavage sites were observed which correspond to the unpaired loop region formed by U20, C21, and U22 (Fig. 6A, lane 4). In both aptamers, the cleavage at U32 is almost abolished in the presence of 5 mM of cognate amino acid (Fig. 6A, lanes 6 and 7). Lead (II) cleavage sites have allowed identification of binding sites for bivalent metal ions, in particular magnesium ion binding sites in RNA in cases where increasing concentrations of Mg^{2+} abolished lead cleavage (43). To test the influence of Mg^{2+} on the lead cleavage at U32 we performed a titration with increasing Mg^{2+} concentrations. No decrease in the cleavage intensity at U32 can be detected (Fig. 6B), suggesting that the cleavage site at position U32 is not due to specific coordination of Pb^{2+} by the aptamers. To examine the possibility that cleavage at this position occurs in a catalytic fashion we divided both aptamers into two halves and designated the 'upper strand' in which the cleavage occurs as 'substrate'. Figure 6C shows the result of the incubation of the 'upper strand' with Pb^{2+} in the absence (lanes 1 and 2) and presence of the 'lower strand' (lanes 3 and 4). Lead-induced cleavage at U32 was found in the upper strand whether or not the lower strand was present. This result clearly shows that cleavage at U32 is not due to specific coordination of Pb^{2+} but rather to a hypersensitive site between U32 and A33.

While a clear protection from cleavage in the presence of the cognate amino acid was observed in the full-length aptamer, this effect was not found in the 'upper strand' construct in which binding of the amino acid does not happen (data not shown). The amino acid-induced protection further supports our suggestion that a conformational change in the aptamer takes place upon amino acid binding. Alternatively, the binding of the amino acid might inhibit the access of the lead-ion to the hypersensitive site. In any case, the fact that cleavage at a hypersensitive site in a functional RNA can be inhibited by a bound ligand might have implications for RNA evolution. This mechanism might have allowed those functional RNAs containing hypersensitive sites that were at a disadvantage to their competitors, which did not contain such sites, to compete more successfully during evolution.

NMR titration of amino acid binding

Imino-proton spectra of the citrulline and arginine aptamers in the absence and in the presence of increasing amounts of their cognate amino acid are shown in Figure 7.
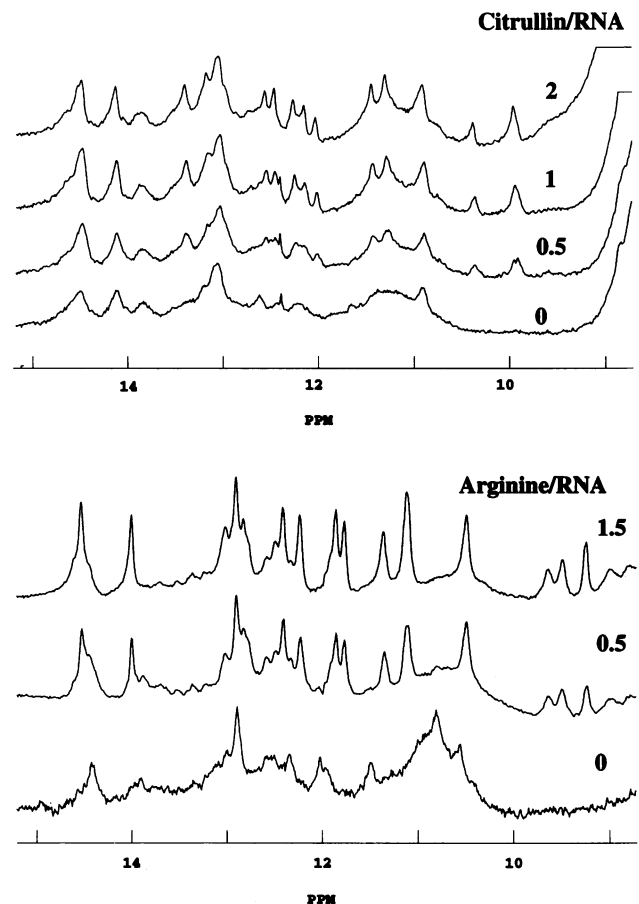
Imino-protons of base paired U and G nucleotides are expected to resonate in the range between 12 and 15 p.p.m. whereas signals corresponding to unpaired imino protons usually appear at ~11 p.p.m. (45). For both aptamers, in the absence of amino acid, several base pairs seem to be formed as indicated by the presence of imino peaks between 12 and 15 p.p.m., but several imino-protons are in an unbound state as indicated by the presence of a broad massif at ~11 p.p.m. This is in qualitative agreement with the secondary structure proposed for the RNAs on the basis of the artificial phylogeny generated by the selection experiments and of the footprinting data presented here. In the absence of amino acid, most of the resonances of the hydrogen
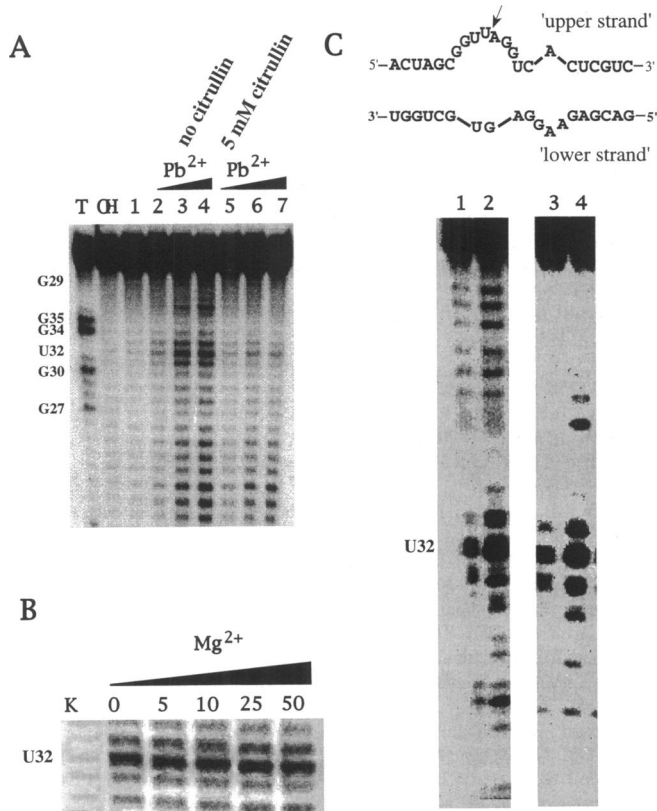
Figure 6. Pb(II)-induced hydrolysis of 5′ ^{32}P-end-labeled RNA 44Cit11.
(A) The cleavage in presence of different concentrations of Pb^{2+}. T, partial
RNase T1 digest; OH, alkaline hydrolysis ladder; lane 1, unmodified RNA;
lanes 2, 3 and 4, free RNA incubated with 0.2, 0.5 and 1 mM Pb(OAc)$_2$,
respectively; lanes 5, 6 and 7, RNA incubated with 0.2, 0.5 and 1 mM
Pb(OAc)$_2$, respectively, in the presence of 5 mM of cognate amino acid. (B) The
magnesium competition of the lead cleavage at U32 in the presence of 1 mM
Pb(OAc)$_2$ and concentrations of Mg^{2+} varying from 0 to 50 mM. K indicates
the unmodified RNA. In (C) the sequence of the synthesized 'upper' and
'lower' strand is shown. The arrow indicates the lead cleavage site at U32. The
gel presents the cleavage of the 'upper strand' at U32. Lanes 1 and 2, incubation
of 'upper strand' in the absence of 'lower strand' with 1 mM Pb(OAc)$_2$ for 1
and 15 h, respectively; lanes 3 and 4, incubation of 'upper strand and 'lower
strand' in the ratio 1:1 with 1 mM Pb(OAc)$_2$ for 1 and 15 h, respectively.

Figure 7. Imino-proton spectra of the citrulline and arginine aptamers
(Cit.NMR and Arg.NMR; see Table 1 for the nucleotide sequence of the RNAs).
Experimental conditions: 1 mM RNA, 20 mM NaCl, T = 5°C, pH 6.8. The ratio
of amino acid to RNA is indicated above each spectrum. Spectra were acquired
in 90% H$_2$O, 10% D$_2$O with a jump-and-return water suppression pulse (44)
on a home-built 360 MHz spectrometer.

bonded imino-protons are broad due to chemical exchange of
these protons with solvent protons. This demonstrates the low
stability of the base pairs formed, which is not surprising because
of the relatively short stems of contiguous base pairs flanking the
poorly structured bulged regions in the constructs used for the
NMR experiment. Upon addition of amino acid, however, the
resonances of the paired imino-protons become sharper. This
effect demonstrates the stabilization of some of the preexisting
base pairs. Furthermore, new signals appear at ~11 p.p.m.,
demonstrating that some of the imino-protons of the bulged
nucleotides which were previouly exposed to the solvent are now
protected from chemical exchange with water protons. This is
again in full agreement with the footprinting experiment performed
in the presence of amino acid and the conformational changes
proposed from the lead cleavage data.

The absence of any substantial differences between the 1:1 and
the 2:1 citrulline to RNA spectra strongly suggest a 1:1
stoichiometry for the complex. The same also holds for the
arginine complex. For both aptamers, >80% of the RNA appeared
to be complexed at a concentration of 1 mM and a 1:1 ratio of
RNA:amino acid, as judged from a comparison of the peak
integrals for the 1:1 and 1:2 complexes. This provides an estimate
for the binding constant of <50 µM in our experimental
conditions. Finally, the complex is in slow exchange at the NMR
time scale as indicated by the presence of two sets of resonances
for the protons of the amino acid (data not shown).

CONCLUSION

The chemical modification experiments of the free L-citrulline
and L-arginine binding aptamers mainly confirm the formation of
the internal loop consisting of the conserved 10mer and 6mer
consensus sequences. The most significant difference of the
proposed secondary structure model concerns the initially
proposed base pairs G29-U13 (Cit) G30-C13 (Arg). These
positions are accessible for reaction with modifying agents. They
are protected in the presence of the ligand, and modification at this
position interferes in the damage selection. Position 13, a U in
64Cit11 and a C in 64Arg11, is one of the three mutations
identified by *in vitro* evolution (4) where the arginine aptamer
differs from the citrulline aptamer. This fact, together with the

observation that the N3 position in U13 (Cit) and C13 (Arg), respectively, is required for binding makes these bases particularly attractive to propose a direct contact to the ligand: N3 in C13 (Arg) is a hydrogen bond acceptor group, whereas in U13 (Cit) it is a hydrogen bond donor. The main difference between citrulline and arginine is that the former contains a hydrogen bond acceptor group in the form of the oxygen atom of the urea moiety whereas the latter carries a hydrogen bond donor in the guanidino-NH_2 group.

Binding of the amino acid not only induces conformational changes, as corroborated by the hypersensitivity of A8 to modification with DMS in the presence of amino acid as well as by the protection of the lead cleavage site at U32 and NMR titration data, but might also stabilize extensive non-canonical base pairing and tertiary interactions within the unpaired loop regions. We conclude this from the results of the footprinting experiments and the damage selection which show that nearly every base within the two consensus sequences is required for binding. In order to understand the structural details of the tertiary interactions proposed, it will be neccessary to carry out a detailed NMR analysis. In the context of such a study, the chemical modification analysis described here will prove to be helpful to obtain a detailed picture of the solution structure of the aptamer–amino acid complexes.

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REFERENCES

1 Famulok,M. and Szostak,J.W. (1992) *J. Am. Chem. Soc.*, **114**, 3990–3991.
2 Connell,G.J., Illangesekare,M. and Yarus,M. (1993) *Biochemistry*, **32**, 5497–5502.
3 Majerfeld,I. and Yarus,M. (1994) *Struct. Biol.*, **1**, 287–292.
4 Famulok,M. (1994) *J. Am. Chem. Soc.*, **116**, 1698–1706.
5 Sassanfar,M. and Szostak,J.W. (1993) *Nature*, **364**, 550–553.
6 Burgstaller,P. and Famulok,M. (1994) *Angew. Chem. Int. Ed. Engl.*, **33**, 1084–1087.
7 Lauhon,C.T. and Szostak,J.W. (1995) *J. Am. Chem. Soc.*, **117**, 1246–1257.
8 Lorsch,J.W. and Szostak,J.W. (1994) *Biochemistry*, **33**, 973–982.
9 Huizenga,D.E. and Szostak,J.W. (1995) *Biochemistry*, **34**, 656–665.
10 Jenison,R.D., Gill,S.C., Pardi,A. and Polisky,B. (1994) *Science*, **263**, 1425–1429.
11 Wang,Y. and Rando,R.R. (1995) *Chem. Biol.*, **2**, 281–290.
12 Lato,S.M., Boles,A.M. and Ellington,A.D. (1995) *Chem. Biol.*, **2**, 291–303.

13 Wallis,M.G., von Ahsen,U., Schroeder,R. and Famulok,M. (1995) *Chem. Biol.*, **2**, 543–552.
14 Ellington,A.D. and Szostak,J.W. (1990) *Nature*, **346**, 818–822.
15 Ellington,A.D. and Szostak,J.W. (1992) *Nature*, **355**, 850–852.
16 Morris,K.N., Tharasow,T.M., Julin,C.M., Simons,S.L., Hilvert,D.L. and Gold,L. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 13028–13032.
17 Prudent,J.R., Uno,T. and Schultz,P.G. (1994) *Science*, **264**, 1924–1927.
18 Joyce,G.F. (1994) *Curr. Opin. Struct. Biol.*, **4**, 331–336.
19 Klug,S.J. and Famulok,M. (1994) *Mol. Biol. Rep.*, **20**, 97–107.
20 Gold,L. (1995) *J. Biol. Chem.*, **270**, 13581–13584.
21 Gold,L., Polisky,B., Uhlenbeck,O., Yarus,M. (1995) *Annu. Rev. Biochem.*, **64**, 763–797.
22 Bock,L.C., Griffin,L.C., Latham,J.A., Vermaas,E.H. and Toole,J.J. (1992) *Nature*, **355**, 564–566.
23 Wang,K.Y., McCurdy,S., Shea,R.G., Swaminathan,S. and Bolton,P.H. (1993) *Biochemistry*, **32**, 1899–1904.
24 Macaya,R.F., Schulze,P., Smith,F.W., Roe,J.A. and Feigon,J. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3745–3749.
25 Schultze,P., Macaya,R.F. and Feigon,J. (1994) *J. Mol. Biol.*, **235**, 1532–1547.
26 Jensen,K.B., Green,L., MacDougal-Waugh,S. and Tuerk,C (1994), *J. Mol. Biol.*, **235**, 237–247.
27 Ehresmann,C., Baudin,F., Mougel,M., Romby,P, Ebel,J.-P. and Ehresmann,B. (1987) *Nucleic Acids Res.*, **15**, 9109–9128.
28 Davanloo,P., Rosenberg,A.H., Dunn,J.J. and Studier,F.W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2035–2039.
29 Milligan,J.F. and Uhlenbeck,O.C. (1989) *Methods Enzymol.*, **180**, 51–63.
30 Stern,S., Moazed,D. and NollerH.F. (1988) *Methods Enzymol.*, **164**, 481–489.
31 Sampson,J.R., Sullivan,F.X., Behlen,L.S., DiRenzo,A.B. and Uhlenbeck,O.C. (1987) *Cold Spring Harbor Symp. Quant. Biol.*, **52**, 267–277.
32 Behlen,L.S., Sampson,J.R., DiRenzo,A.B. and Uhlenbeck ,O.C. (1990) *Biochemistry*, **29**, 2515–2523.
33 Krzyzosiak,W.J., Marciniec,T., Wiewiorowski,M., Romby,P., Ebel,J.P. and Giege,R. (1988) *Biochemistry*, **27**, 5771–5777.
34 Marciniec,T., Ciesiolka,J., Wrzesinski,J. and Krzyzosiak,W.J. (1989), *FEBS Lett.*, **243**, 293–298.
35 Ciesiolka,J., Wrzesinski,J., Gornicki,P., Podkowinski,J. and Krzyzosiak,W.J. (1989) *Eur. J. Biochem.*, **186**, 71–77.
36 Pan,T., Gutell,R.R. and Uhlenbeck,O.C. (1991) *Science*, **254**, 1361–1364.
37 Otzen,D.E., Barciszewski,J. and Clark,B.F. (1993) *Biochem. Mol. Biol. Int.*, 31, 95–103.
38 Zietkiewicz,E., Ciesiolka,J., Krzyzosiak,W.J. and Slomski,R. (1989) *Nuclear Structure and Function*, Plenum Publishing Corporation, New York, pp. 453–457.
39 Gornicki,P., Baudin,F., Romby,P., Wiewiorowski,M., Krzyzosiak,W.J., Ebel,J.P., Ehresmann,C., and Ehresmann,B. (1989) *J. Biomol. Struct. Dyn.*, **6**, 971–984.
40 Zito,K., Hüttenhofer,A., and Pace,N.R. (1993), *Nucleic Acids Res.*, **21**, 5916–5920.
41 Ciesiolka,J., Hardt,W.D., Schlegel,J, Erdmann,V.A. and Hartmann,R.R. (1994) *Eur. J. Biochem.*, **219**, 49–56.
42 Ciesiolka,J., Lorenz,S. and Erdmann,V.A. (1992) *Eur. J. Biochem.*, **204**, 575–581.
43 Streicher,B., von Ahsen,U. and Schroeder,R. (1993) *Nucleic Acids Res.*, **21**, 311–317.
44 PlateauP. and Guéron,M. (1982) *J. Am. Chem. Soc.*, **104**, 7310–7311.
45 Varani,G. and Tinoco,I.,Jr. (1991) *Q. Rev. Biophys.*, **24**, 479–532.