Pokeweed antiviral protein (PAP) is a ribosome-inactivating protein that catalytically cleaves a specific adenine base from the highly conserved α-sarcin/ricin loop of the large ribosomal RNA, thereby inhibiting protein synthesis at the elongation step. Recently, we discovered that alanine substitutions of the active center cleft residues significantly impair the depurinating and ribosome inhibitory activity of PAP. Here we employed site-directed mutagenesis combined with standard filter binding assays, equilibrium binding assays with Scatchard analyses, and surface plasmon resonance technology to elucidate the putative role of the PAP active center cleft in the binding of PAP to the α-sarcin/ricin stem loop of rRNA. Our findings presented herein provide experimental evidence that besides the catalytic site, the active center cleft also participates in the binding of PAP to the target tetraloop structure of rRNA. These results extend our recent modeling studies, which predicted that the residues of the active center cleft could, via electrostatic interactions, contribute to both the correct orientation and stable binding of the substrate RNA molecules in PAP active site pocket. The insights gained from this study also explain why and how the conserved charged and polar side chains located at the active center cleft of PAP and certain catalytic site residues, that do not directly participate in the catalytic deacylenylation of ribosomal RNA, play a critical role in the catalytic removal of the adenine base from target rRNA substrates by affecting the binding interactions between PAP and rRNA.
analysis of binding using surface plasmon resonance (SPR) biosensor technology. Here, we present direct evidence that, compared with wild-type PAP, both FLP-4 and FLP-7 exhibit significantly impaired affinity for RNA substrates, providing a cogent explanation for their reduced depurinating activity. Similarly, FLP-9 (Ala122Ala123) with alanine substitutions expected to affect the orientation of the substrate adenine in the active site showed markedly reduced affinity for RNA targets. Catalytic site mutants FLP-12 (Ala179) and FLP-13 (Ala208), that were included as controls, also showed markedly reduced binding affinity for RNA substrates, consistent with the previously established importance of Arg179 and Thr209 residues in RNA binding and depurination (10, 17, 18). By comparison, recombinant PAP mutants with alanine substitutions of residues 28K29 (FLP-1) and 111SR112 (FLP-8) that are distant from the active site and active center cleft showed normal binding affinity to RNA substrates. The experimental findings presented herein are in accordance with the predictions of our recent modeling studies, which suggested that the active center cleft of PAP is important for binding to RNA.

MATERIALS AND METHODS

Molecular Modeling—The molecular model of the PAP-ribosome (large subunit) complex was derived from the 2.4 Å crystal structure of Holoarcus marismortui (Protein Data Bank access code 1FFK) (19) and the crystal structure of PAP-nucleotide complex (access code 1poo). This model represents a refinement of our recently published model of the PAP-RNA stem loop complex (16). First, we superimposed the H. marismortui subunit ribosome with the stem loop structure in our previous PAP-RNA complex model. The conformation of RNA was adjusted around the adenine A2697 (A2660 in E. coli) (Escherichia coli) of the GAGA tetraloop, and the rest of the PAP molecule remains unchanged. The adenine was manually adjusted by a 9° out-flipping rotation at the C5′ position and a 5.4 Å translation of the adenine ring. The model was used to perform fixed docking using the Docking module in InsightII employing the CVFF (29). The parameters used in this docking included searching for five unique structures: 1000 minimization steps for each structure, energy range of 10.0 kcal/mol, maximum translation of the ligand of 3.0 Å, maximum rotation of the ligand of 10°, and an energy tolerance for 1500 kcal/mol. During the minimization steps of the docking procedure, only the tetraloop and the active site residue of PAP were allowed to be flexible, whereas the rest of RNA and PAP remained fixed. The refined structure was then analyzed in CHAIN (20).

Construction of Mutants—Recombinant wild-type PAP construct (pBS-PAP) was obtained by subcloning the PAP-I gene encoding PAP amino acid residues 1–259 of the BoxIII sites of the expression vector, pBluescript SK (Strategene, La Jolla, CA). PAP mutants were constructed using site-directed mutagenesis techniques as described previously (16). Unlike native PAP, which has 262 amino acids, recombinant PAP proteins have 292 amino acids. Therefore, they are larger than native PAP (33 versus 29 kDa) (16).

Expression and Purification of Mutants—Wild-type and mutant recombinant PAP proteins were expressed in E. coli M15190 as inclusion bodies, isolated, solubilized, and refolded, as described previously (21). The refolded proteins were analyzed by SDS-12% polyacrylamide gel electrophoresis. Protein concentrations were determined from the gel using bovine serum albumin (BSA) as a standard.

Immunoblot Analysis of PAP Mutants—One μg of the protein samples was resolved on a SDS-12% polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Roche Molecular Biochemicals) using the Bio-Rad transblot apparatus, as described previously (21). The membrane was immunooblotted using rabbit anti-PAP serum (Sigma; 1:1000 dilution) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma; 1:1000 dilution) as the primary and secondary antibodies, respectively. The blot was developed using 3,3′-diaminobenzidine (Sigma) as the colorimetric indicator for peroxidase activity.

Adenine Release Assays—The release of adenine from E. coli 23 S/16 S ribosomal RNA (Roche Molecular Biochemicals) was measured using HPLC (Hewlett Packard, Palo Alto, CA) equipped with a diode array detector, as described previously (16).

Preparation of Radiolabeled RNA Substrates—A 27-mer oligoribonucleotide (5′-CCGGCUCCAGGAGGAGAACCCCGAGG-3′), which contains the required substrate tetraloop motif, GAGA (α-mannin/ricin loop), was synthesized on the 1-μmol scale and HPLC-purified by CyberSyn (Lennan, PA). The oligoribonucleotide (oligo) (5 μg) was labeled at the 5′-end using γ-32P-ATP and T4 polynucleotide kinase (Roche Molecular Biochemicals), and the unincorporated label was removed by Sephadex G-25 quick spin oligo columns (Roche Molecular Biochemicals). The resulting labeled oligo was dissolved in water (1 μCi/ml) for 10 min at room temperature. The oligo was purified on a Sephadex G-25 spin column (Sigma) as the colorimetric indicator for peroxidase activity.

The E. coli 23 S/16 S ribosomal RNA (30 μg) was alkaline-hydrolyzed by treating with 500 μl (final volume) of 100 mM NaHCO3, pH 9.5, and 1% Na2CO3 at 95 °C for 15 min. Following incubation on ice for an additional 10 min, the reaction was terminated by adding 50 μl of 7.5 mM ammonium acetate, and the RNA was recovered by extraction with phenol/chloroform (1:1) and precipitated with ethanol. The RNA pellet was dissolved in the labeling reaction mixture containing 2.0 μl (100 pmol/m) of γ-32P-ATP, 10 μl of 10X polynucleotide kinase buffer (0.1X MgCl2, 50 mM dithiothreitol, 50% glycerol, 0.5X Tris, pH 9.5), 5 units of polynucleotide kinase, and the volume was adjusted to 40 μl with RNase-free water. After 30 min of incubation at 37 °C, the unincorporated label was removed by Sephadex G-50 quick spin RNA columns (Roche Molecular Biochemicals) per the supplier's instructions. The specific activities of the labeled oligoribonucleotide and RNA were 0.73 and 0.9 μCi/μg, respectively. Typically, 45–55% of the γ-32P-ATP was incorporated into the labeled oligonucleotide.

Filter Binding Assays—For saturation binding assays, radiolabeled 23 S/16 S RNA (3.5 pmol; specific activity 460 cpm/pmol), radiolabeled RNA SR loop oligo (75 pmol; specific activity 140 cpm/pmol), or radiolabeled control oligo (75 pmol; specific activity 172 cpm/pmol) were preincubated in separate tubes at 65 °C for 5 min in 50 μl of 350 mM KCl, 20 mM MgCl2, 30 mM Tris-HCl, pH 7.5. Increasing amounts of recombinant wild-type or mutant PAP proteins were mixed with the preincubated radiolabeled probes, and the solution was adjusted to 150 μl with binding buffer (1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 3 mM MgCl2, 0.01% BSA, and 10 mM HEPES, pH 8.0). The mixture was incubated for another 15 min at 30 °C and then placed on ice for 10 min. The reaction mixture was filtered through a nitrocellulose membrane (Millipore Corp.; type HA, 0.45-μm pore size, 25-mm diameter), and the membrane was washed three times with 1 ml of wash buffer (1 mM EDTA, 0.1% Tween 20, and 10 mM HEPES, pH 8.0) each time. The membrane was dried at room temperature and was counted for 32P radioactivity.

Substrate specific binding of wild-type and mutant PAP proteins was determined by treating 25 pmol of protein samples with increasing concentrations of 32P-labeled RNA SR loop oligo (specific activity 140 cpm/pmol) for 30 min at room temperature in a final volume of 150 μl with binding buffer. The reaction mixture was filtered through a nitrocellulose membrane, washed, and counted as described previously (22). The association plot (22) analysis of equilibrium binding data was performed as described previously (23). The association constant Ka was calculated from the slope of the line.

Competition binding was performed by incubating 33 pmol of wild-type PAP or BSA (control) proteins with 50 pmol of 32P-labeled SR loop oligo and increasing concentrations (0–1500 pmol) of unlabeled SR loop oligo as competitor in 150 μl of binding buffer. The reaction mixture was incubated at room temperature for 1 h, and the bound oligo was separated from the bound oligo by filtration and washing through a nitrocellulose membrane. The membrane was dried at room temperature, and the radioactivity in the membrane was measured as described before. Each experiment was repeated at least two times, and the average values were plotted using the CA-CCRICKET Graph III application program.

SPR Analysis—A BiAcore 2000 SPR-based biosensor system (Amersham Pharmacia Biotech) was used to measure the kinetic parameters of the interaction between soluble mutant PAP proteins (analytes) and the immobilized RNA oligo (ligand). The oligo (27-mer) was synthesized using HPLC-purified T4 polynucleotide kinase (CyberSyn, Lennan, PA). The 5′-end biotinylated oligo (40 μg/ml) was immobilized (streptavidin-biotin coupling) on the surface of the streptavidin (SA) sensor chips by injecting 30 μl of the oligo at a flow rate of 5 μl/min in HBS-EP buffer (0.1 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% polysorbate 20). Typically, the response unit increase following this procedure was 70–100. The unoccupied SA surface was blocked by injecting 30 μl of 25 μg/ml biotin in HBS-EP buffer as above. All of the
proteins were dissolved in HSEM buffer (10 mM HEPES, pH 8.0, 50 mM NaCl, 1 mM EDTA, 5 mM MgCl₂) to yield a final concentration of 5 μg/ml. In a kinetic study, 30-μl samples (150 nM) were injected at 25 °C at a flow rate of 8 μl/min onto the sensor chip surface on which the oligo has been immobilized or onto a control surface on which SA had been blocked with biotin, using HBS-EP as the running buffer. Between samples, the binding surfaces were regenerated by a 3-min injection of 2 mM NaCl at a flow rate of 10 μl/min. Due to the large number of samples, the experiment was carried out twice on the same chip, and samples were injected in random order. To prepare the data for analysis, base lines were adjusted to zero for all curves, and injection start times were aligned. Background sensorgrams were then subtracted from the experimental sensorgrams to yield curves representing specific binding. The association and dissociation phases of the sensorgrams were fit simultaneously, assuming a simple bimolecular reaction model for interaction between soluble analyte and immobilized ligand, equivalent to the Langmuir isotherm for adsorption to a surface. The goodness of fit was assessed by inspecting the statistical value \( \chi^2 \) and the residuals observed – calculated. The \( \chi^2 \) values were low (≤2), and the residuals were randomly distributed about zero. The association and dissociation rate constants were calculated by nonlinear fitting of the primary sensorgram data using the BIAevaluation software (version 3.0), supplied with the instrument (BIAcore Inc.). Affinities were calculated from rate constants and from analysis of equilibrium binding.

RESULTS AND DISCUSSION

Molecular Model of PAP-Ribosome (Large Subunit) Complex and Structure-based Design of Recombinant PAP Proteins with Altered rRNA Binding Affinity—Our previous modeling studies (16) indicated that the active center cleft residues Asn⁶⁹ and Asn⁷⁰, and Asp⁹² as well as the active site residue Arg¹²², which are not directly involved in the catalytic depurination of rRNA, promote specific interactions with the phosphate backbone of the target α-sarcin/ricin stem loop of rRNA. Therefore, mutations of these residues were predicted to result in destabilization of interactions with rRNA. Our refined molecular model of the PAP-rRNA stem loop complex is depicted in Fig. 1. According to this model, PAP residues 43, 67 (paired with 97), 69–70, 92, 206–210, 212–213, 217, 224–225, and 253–255 participate in its interaction with rRNA. The mutant PAP protein FLP-4 (⁹⁰⁹²⁹²) has been engineered by alanine substitution of the active center cleft residues Asn⁹⁰ and Asn⁹², which are located on an antiparallel \( \beta \) turn and interact via hydrogen bonds with the backbone of rRNA from the A¹⁻³ position to the A¹⁻⁷ position. FLP-7 (⁹⁰⁹²⁹²) has been engineered by alanine substitution of the active center cleft residues 90–92 (⁹⁰⁹²⁹²), which are positioned on the C-terminal of the \( \beta \) strand and are predicted to contribute to the binding of PAP to the tetraloop structure of RNA. Asp⁹² interacts with the base of G¹⁻¹, Phe⁹⁰ has van der Waals interactions with four nearby hydrophobic residues. Thus, mutation of Phe⁹⁰ is anticipated to affect the local conformation of the \( \beta_6 \) strand and the following loop region, which consists of residues 121–123 (¹²²⁴¹²³). The 121–123 loop interacts with the sugar, base, and phosphate groups of the targeted adenosine. Therefore, both FLP-4 and FLP-7 were predicted to have impaired binding to rRNA. The catalytic site mutant FLP-9 (¹²²⁴¹²³) has alanine substitutions of Arg¹²² and Tyr¹²³. Arg¹²² interacts with the phosphate of the targeted adenosine, and the base ring of Tyr¹²³ stacks...
with the targeted adenine base. Therefore, FLP-9 was predicted to have a poor binding affinity to rRNA and poor enzymatic activity. By contrast, the control PAP mutants FLP-1 (28AA29) and FLP-8 (111AA112) with mutations of residues 28KD29 and 111SR112 that are distant from the rRNA binding site were predicted to have a normal rRNA binding comparable with that of wild-type PAP. These predictions are in accord with the recently reported rRNA depurinating activities of FLP-1, FLP-4, FLP-7, FLP-8, FLP-9, FLP-12 (Ala179), and FLP-13 (Ala208) (16). The rRNA depurinating activities of the active center cleft mutants FLP-4 and FLP-7 were significantly lower than the activity of wild-type PAP. By comparison, the depurinating activities of FLP-1 and FLP-8 were comparable with that of wild-type PAP (Table I).

**Table I**

Sequence identity, adenine release, and substrate binding of recombinant wild-type and mutant PAP proteins

| Mutant   | Original residues | Substituted residues | Adenine released | Maximum bound^b | rRNA Oligo |
|----------|-------------------|----------------------|------------------|-----------------|------------|
| Wild type| 28KD29            | 28AA29               | 296 ± 15         | 0.79 ± 0.03     | 1.2 ± 0.2  |
| FLP-1    | 69NN70            | 69AA70               | 132 ± 12         | 0.27 ± 0.02     | 0.53 ± 0.1 |
| FLP-4    | 90FND92           | 90AAA92              | 19 ± 5           | 0.29 ± 0.02     | 0.38 ± 0.1 |
| FLP-7    | 111SR112          | 111AA112             | 376 ± 11         | 0.75 ± 0.03     | 1.2 ± 0.1  |
| FLP-9    | 122RY123          | 122AA123             | 12 ± 3           | 0.29 ± 0.02     | 0.47 ± 0.1 |
| FLP-12   | Arg179            | Ala179               | 14 ± 7           | 0.28 ± 0.02     | 0.42 ± 0.1 |
| FLP-13   | Trp208            | Ala208               | 4 ± 2            | 0.27 ± 0.02     | 0.48 ± 0.1 |

^a The adenine released values were adopted from Rajamohan et al. (16) for comparison.

^b The maximum level of binding (Bmax) of 32P-labeled 23 S/16 S rRNA and 32P-labeled SR loop oligo (pmol/pmol of protein) were derived from fitting the data of Fig. 4, A and C, to a hyperbolic binding function (Graphpad Prism 2). Each value is the mean and S.D. of three experiments.
refolded, and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2A). Each of the mutant PAP proteins had an apparent molecular mass of 33 kDa, similar to that of the recombinant wild-type PAP (Fig. 2A). The refolded recombinant wild-type and mutant proteins were highly immunoreactive with the anti-PAP serum (Fig. 2B).

We first evaluated the saturation binding of radiolabeled 23 S/16 S rRNA (Fig. 3A) with increasing concentrations of recombinant wild-type or mutant PAP proteins with alanine substitutions in three independent experiments using standard filter binding assays. When increasing amounts of PAP proteins were reacted with 3.5 pmol of 32P-labeled 23 S/16 S rRNA (specific activity 460 cpm/pmol), wild-type PAP showed concentration-dependent and saturable binding to the substrate RNA (23 S/16 S rRNA). As evidenced in Fig. 4A and Table I, the catalytic site mutants FLP-9(122AA123), FLP-12(A179), and FLP-13(A208) as well as the active center cleft mutants FLP-4 (69AA70) and FLP-7 (90AAA92) exhibited significantly impaired RNA binding ability when compared with wild-type PAP. By comparison, recombinant PAP mutants with alanine substitutions of residues 28KD29 (FLP-1) and 111SR112 (FLP-8) that are distant from the catalytic site and active center cleft were able to bind 23 S/16 S rRNA in amounts comparable with that of the wild-type PAP (Fig. 4A, Table I). Control protein BSA did not bind to 32P-labeled 23 S/16 S rRNA even at the highest concentration.

Interaction of Recombinant PAP Mutants with α-Sarcin/Ricin Stem Loop of Eukaryotic 28 S rRNA—It is known that PAP catalytically removes a specific adenine from the highly conserved, surface-exposed, α-sarcin/ricin loop in the large rRNA of prokaryotic and eukaryotic ribosomes (3–6). Therefore, in our present study of RNA-PAP interactions, we used a 27-mer model RNA fragment corresponding to the conserved α-sarcin/ricin stem loop (SR loop) of eukaryotic 28 S rRNA (Fig. 3B). Synthetic RNA fragments that mimic the local structures of rRNA have widely been employed to investigate RNA-protein interactions (24–26). The 5′-end of this oligoribonucleotide (Fig. 3C) was radiolabeled with 32P (specific activity 140 cpm/pmol), and its interaction with increasing amounts of wild-type or mutant recombinant PAP proteins was investigated using filter binding assays. We first examined the binding of PAP (33 pmol) to 32P-labeled SR loop oligo (50 pmol) in the absence as well as presence of increasing concentrations of unlabeled oligo. PAP exhibited significant binding to the oligo, and this binding was competitively blocked by the cold oligo in a concentration-dependent fashion, confirming the specificity of the binding interaction (Fig. 4B). Control protein BSA, however, exhibited very little binding, and its binding was not competitively blocked by the unlabeled oligo (Fig. 4B).

We next compared the binding of wild-type and mutant PAP proteins to the SR loop oligo. In accordance with the binding results obtained using naked 23 S/16 S rRNA, the active center cleft mutants, FLP-4 (69AA70) and FLP-7 (90AAA92), as well as the catalytic site mutants FLP-9 (122AA123), FLP-12 (A179), and FLP-13 (A208), exhibited significantly reduced binding to the stem loop RNA fragment (Table I, Fig. 4C). By comparison, the binding of recombinant PAP mutants FLP-1(A28AA29) and FLP-8 (111AA112) to the SR loop RNA fragment is comparable with that of the wild-type PAP (Fig. 4C, Table I). BSA, which was included as a non-RNA-binding control protein, showed no binding to the SR loop oligo.

We next performed equilibrium binding studies to determine the maximum specific substrate (SR loop oligo) binding capacity (Bmax) and apparent association constant (Ks) of the equilibrium binding to the stem loop RNA fragment for wild-type and the mutant recombinant PAP proteins. Recombinant PAP proteins (25 pmol) were incubated with increasing concentrations of the 32P-labeled SR stem loop RNA fragment for 30 min at room temperature. All PAP proteins exhibited saturable

![Figure 4](image-url)
binding at increasing substrate oligoribonucleotide concentrations (Fig. 5A). For each recombinant PAP protein, the display of the equilibrium binding data in a Scatchard coordinate system yielded a straight line \((r = -0.99)\) for binding measured over a concentration range of 0–22.5 pmol, supporting the notion that a single class of rRNA binding sites exists for PAP (Fig. 5B). The intercepts on the x axis \((B_{\text{max}})\) that correspond to the maximum loop RNA fragment binding capacity of the PAP proteins were 21 ± 4 pmol/µg for recombinant wild-type PAP, 13 ± 2 pmol/µg for FLP-4 \((\text{Ala} 25)\), 13 ± 2 pmol/µg for FLP-7 \((\text{Ala} 99)\), 14 ± 2 pmol/µg for FLP-9 \((\text{Ala} 122)\), 14 ± 3 pmol/µg for FLP-12 \((\text{Ala} 179)\), and 12 ± 2 pmol/µg for FLP-13 \((\text{Ala} 208)\) (Fig. 5, B and C). These findings demonstrate that RNA binding capacity of PAP is markedly reduced by mutations involving its catalytic site or active center cleft. The estimated values of the apparent \(K_a\) as determined from the negative slope of the linear Scatchard plots were 61 ± 12 pmol/µg for the wild-type protein, 158 ± 11 pmol/µg for FLP-4, 148 ± 14 pmol/µg for FLP-7, 138 ± 10 pmol/µg for FLP-9, 142 ± 15 pmol/µg for FLP-12, and 132 ± 9 pmol/µg for FLP-13 (Fig. 5C). Thus, the \(K_a\) values of the mutant PAP proteins were higher than the \(K_a\) of the wild-type protein. Therefore, the reduced binding capacity of the mutant PAP proteins to the stem loop RNA fragment appears to be caused at least in part by an impairment in the association phase of the rRNA binding to the mutated binding region.

In order to further assess the binding specificity, we also used a \(^{32}\)P-labeled control oligo \((5’-\text{CGCGCCUUUUCGCCGCGG-3’})\) that does not contain the required substrate tetraloop motif, GAGA, for PAP binding. Filter binding assays with this control oligo did not exhibit significant binding to either the wild-type or mutant PAP proteins (Fig. 5D). These experiments further confirm that PAP proteins specifically bind to their RNA substrates via the target tetraloop. Taken together, the presented results of the saturation and competition binding assays confirmed the predictions of our modeling studies (16) and provided the first direct biochemical evidence that, besides the catalytic site residues of PAP, the residues of the active center cleft located between the central and C-terminal domains of PAP also contribute to its binding to the target \(\alpha\)-sarcin/ricin stem loop of eukaryotic 28S rRNA.

Because of the inherent uncertainties associated with using radiolabeled substrates in binding assays, we next sought to compare the affinity of recombinant PAP proteins for the non-radiolabeled stem loop RNA fragment by surface plasmon resonance. The active center cleft mutants FLP-4 and FLP-7 as well as the catalytic site mutant FLP-9 had much slower on-rates than the wild-type recombinant PAP protein (Fig. 6, A and B). All three mutant proteins also had faster off-rates, consistent with instability of their RNA binding (Fig. 6B). The affinities of FLP-4 \((K_D = 1.0 \text{ nM})\), FLP-7 \((K_D = 1.4 \text{ nM})\), and FLP-9 \((K_D = 3.3 \text{ nM})\) were 5-, 7-, and 16-fold lower, respectively, than the affinity of the wild-type PAP \((K_D = 0.2 \text{ nM})\) protein.

FIG. 5. Binding of recombinant PAP proteins to radiolabeled oligoribonucleotide as a function of free ligand concentration. Panel A, binding of PAP mutant proteins (25 pmol) with increasing concentrations of \(^{32}\)P-labeled \(\alpha\)-sarcin/ricin loop oligo. Panel B, equilibrium binding data from A was transformed and replotted in the Scatchard plot. Results are shown as number of oligonucleotide specifically bound to 1.0 µg of protein \((B)\) and concentration \((\text{nm})\) of free radiolabeled oligonucleotide \((F)\). Panel C, Scatchard data. Panel D, binding of recombinant PAP proteins (25 pmol) to \(^{32}\)P-labeled control oligo. Data points represent mean values obtained from two independent experiments.

FIG. 6. Binding of wild-type and mutant PAP proteins to a synthetic oligoribonucleotide (27-mer) that mimics the \(\alpha\)-sarcin/ricin domain in 28S rRNA. A, a representative sensorgram showing the binding of wild-type (WT) and mutant PAP proteins to the 5’-biotin-labeled oligoribonucleotide immobilized, 70–100 response units \((RU)\), on a SA sensor chip. 30 µl of each protein (150 nM) was injected at a flow rate of 8 µl/min. B, kinetic rates and dissociation constants of the binding interactions between recombinant PAP proteins and the 27-mer oligoribonucleotide.
(Fig. 6B). By comparison, the affinity of the recombinant PAP mutant FLP-8 with alanine substitution of residues 111SR112 that are distant from the catalytic site and active center cleft for the stem loop RNA fragment was identical to that of the wild-type PAP (Fig. 6B).

In summary, we employed site-directed mutagenesis combined with standard filter binding assays, equilibrium binding assays with Scatchard analyses, and surface plasmon resonance technology to elucidate the putative role of the PAP active center cleft in the binding of PAP to the α-sarcin/ricin stem loop of rRNA. Our findings provide experimental evidence that besides the catalytic site the active center cleft also participates in the binding of PAP to the target tetraloop structure of rRNA. These results extend our recent modeling studies (16), which predicted that the residues of the active center cleft could, via electrostatic interactions, contribute to both the correct orientation and stable binding of the substrate RNA molecules in the PAP active site pocket. The insights gained from this study also explain why and how the conserved charged and polar side chains located at the active center cleft of PAP and certain catalytic site residues that do not directly participate in the catalytic deadenylation of ribosomal RNA play a critical role in the catalytic removal of the adenine base from target rRNA substrates by affecting the binding interactions between PAP and rRNA.

Control mutant proteins FLP-1 and FLP-8 exhibited similar RNA binding as wild-type PAP protein. Furthermore, the expression, solubility, and yield of refolded wild-type and mutant proteins were comparable (see Fig. 2). Nevertheless, the presented findings should be interpreted with due caution because of the inherent shortcomings of the mutagenesis techniques, which may yield mutant proteins with a nonnative conformation, especially when the mutant proteins are insoluble and need to be renatured.

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