Evidence for the importance of electrostatics in the function of two distinct families of ribosome inactivating toxins

ALEXEI V. KORENNYKH,^{1,2} CARL C. CORRELL,^{3,4} and JOSEPH A. PICCIRILLI^{1,2,3}

¹Department of Chemistry, The University of Chicago, Chicago, Illinois 60637, USA

²Howard Hughes Medical Institute, The University of Chicago, Chicago, Illinois 60637, USA

³Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois 60637, USA

⁴Department of Biochemistry and Molecular Biology, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois 60064, USA

ABSTRACT

α -Sarcin and ricin represent two structurally and mechanistically distinct families of site-specific enzymes that block translation by irreversibly modifying the sarcin/ricin loop (SRL) of 23S–28S rRNA. α -Sarcin family enzymes are designated as *ribotoxins* and act as endonucleases. Ricin family enzymes are designated as ribosome inactivating proteins (RIP) and act as N-glycosidases. Recently, we demonstrated that basic surface residues of the ribotoxin restrictocin promote rapid and specific ribosome targeting by this endonuclease. Here, we report that three RIP: ricin A, saporin, and gypsophilin depurinate the ribosome with strong salt sensitivity and achieve unusually fast $k_{\text{cat}}/K_m \sim 10^9\text{--}10^{10} \text{ M}^{-1}\text{s}^{-1}$, implying that RIP share with ribotoxins a common mechanism of electrostatically facilitated ribosome targeting. Bioinformatics analysis of RIP revealed that surface charge properties correlate with the presence of the transport chain in the RIP molecule, suggesting a second role for the surface charge in RIP transport. These findings put forward surface electrostatics as an important determinant of RIP activity.

Keywords: ribosome; electrostatic; kinetics; mechanism; Smoluchowski; ribotoxin

INTRODUCTION

Restrictocin, an α -sarcin-like ribotoxin from fungi, catalyzes endoribonucleolytic cleavage within the sarcin/ricin loop (SRL) of the large ribosomal subunit (Fig. 1A; Wool 1997). Basic surface residues located on restrictocin's active site face mediate Coulomb interactions with the ribosome and enhance the specificity and speed of the ribosome targeting (Korennykh et al. 2006). Electrostatic interactions give several characteristic features to the restrictocin-catalyzed ribosome cleavage reaction that include (1) strong inverse dependence of the ribosome cleavage rate (and binding) on KCl concentration; (2) unusually rapid maximum $k_{\text{cat}}/K_m = 1.7 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$; and (3) multiple

nonspecific restrictocin-binding sites on the ribosomal surface (Korennykh et al. 2006). These binding sites (~ 50) enhance the macroscopic association constant of the ribotoxin by about 50-fold, thereby maintaining saturating (k_{cat}) conditions for restrictocin at in vivo salt and ribosome concentrations. These distinct electrostatic signatures together with the Poisson–Boltzmann calculations (Baker et al. 2001) imply that negative electrostatic potential covers much of the ribosomal surface. The negative electrostatic potential arises from both the negatively charged phosphodiester backbone and from conserved solvent-exposed acidic patches on ribosomal proteins, suggesting evolutionary conservation of the ribosome's overall electrostatics. This raises the possibility that electrostatic interactions may similarly facilitate ribosome targeting by proteins other than the α -sarcin-like ribotoxins.

Here, we test this possibility experimentally with three ribosome-inactivating proteins (RIP) that are N-glycosidases from plants: ricin A-chain, saporin, and gypsophilin. RIP share a common folded structure and catalyze SRL depurination at the adenosine adjacent to the α -sarcin cleavage site, but have no sequence

Reprint requests to: Joseph A. Piccirilli, Department of Biochemistry and Molecular Biology, and the Howard Hughes Medical Institute, The University of Chicago, 929 East 57th Street, CIS 406, Chicago, IL 60637, USA; e-mail: jpccirilli@uchicago.edu; fax: (773) 702-0271; or Carl C. Correll, Department of Biochemistry and Molecular Biology, Rosalind Franklin University of Medicine and Science, 3333 Green Bay Road, North Chicago, IL 60064, USA; e-mail: carl.correll@rosalindfranklin.edu; fax: (847) 578-3240.

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FIGURE 1. Location of the toxin target sites within SRL and comparison of the folded structures and isoelectric points of ribosome inactivating toxins. (A) Tertiary structure of the rat SRL RNA (PDB entry 430D) showing the sites of cleavage by α -sarcin-like ribonucleases and by N-glycosidases. (B) Comparison of the tertiary structures of ribotoxin restrictocin and RIP ricin A. (C) Distribution of calculated isoelectric points within RIP-I and RIP-II toxins. Proteins were grouped in three categories shown by the three bars: acidic ($pI_{calc} \leq 6$), neutral ($6 < pI_{calc} < 8$), and basic ($pI_{calc} \geq 8$). The height of the bars (N) corresponds to the number of RIP found within each group. Isoelectric points were calculated from polypeptide sequences (Materials and Methods).

or structural similarity with the α -sarcin endonucleases (Fig. 1A,B; Supplemental Fig. 1). Therefore, plant N-glycosidases provide suitable probes for testing whether electrostatic interactions are genuine to α -sarcin ribotoxins or may extend more generally to protein–ribosome interactions. For ricin A, the ribosome modification activity decreases upon chemical modifications of Arg residues lying outside the active site cleft (Watanabe et al. 1994) or upon site-directed mutagenesis of surface Arg residues (Marsden et al. 2004), consistent with the contribution of electrostatic contacts to the ricin activity against ribosomes. In light of our recent findings with restrictocin, it is appealing to suggest that ricin A employs these surface charges to target the ribosome via a mechanism similar to that of restrictocin. Accordingly, we analyzed the ribosome depurination reaction catalyzed by ricin A and two additional RIP using the quantitative framework developed previously during restrictocin studies (Korennykh et al. 2006).

RESULTS AND DISCUSSION

As an initial assessment of whether ricin-like N-glycosidases (plant and bacterial RIP) might exploit the electrostatic features of the ribosome, we examined calculated isoelectric points (pI_{calc}) of 96 unique RIP sequences from two RIP types: RIP-I and RIP-II (Supplemental Figs. 2, 3; Gasteiger et al. 2003). RIP-II contains two polypeptide chains, chains A and B, linked by a disulfide bond. The A chain harbors the N-glycosidase activity for SRL depurination and shares sequence, structural homology, and active site composition with single-chain RIP-I toxins (Supplemental Fig. 1). The B chain enables receptor-mediated uptake of RIP-II toxins via retrograde transport along the secretory pathway and release of the catalytically active A chain into the cytoplasm (Wesche 2002; Stirpe 2004; Spooner et al. 2006). Our analysis revealed that RIP-I have isoelectric points predominantly in the basic pH range (Fig. 1C). In contrast, the catalytic A chains of RIP-II have isoelectric points that lie predominantly in the acidic and neutral pH range (Fig. 1C; Supplemental Fig. 2). As RIP-I and A chains of RIP-II share the same tertiary fold and mechanism of action on ribosomes (Marchant and Hartley 1995; Stirpe 2004), this trend apparently reflects adaptation for functional reasons. The significance of this adaptive trait remains unclear (Benner and Ellington 1988), although some toxins that violate this architectural imperative have weak toxic potency. For example, the RIP-I bouganin with pI_{calc} in the acidic range (Supplemental Fig. 2) exhibits poor cellular internalization and apparently has the lowest cytotoxicity among N-glycosidases (den Hartog et al. 2002). Fusion of another RIP-I, momordin ($pI_{calc}=9.3$), to the ricin B-chain results in a chimeric toxin that undergoes inefficient cellular entry and has weaker toxic potency than that of native ricin (Sharma et al. 1999).

Recent studies have revealed significant differences in the cellular uptake routes for RIP-I and RIP-II molecules. Internalization of positively charged RIP-I such as saporin and gelonin occurs in a receptor-independent manner via fluid-phase endocytosis (Sandvig and van Deurs 2005; Vago et al. 2005), whereas receptor-dependent internalization of RIP-II toxins such as ricin or shiga toxin involves retrograde transport to the endoplasmic reticulum (ER), where protein disulfide isomerase (PDI) activates the toxins by releasing the B chain (Spooner et al. 2006). The differences between the two RIP types may reflect functional adaptation to their uptake routes. For example, proteins in the ER tend to have acidic pI values (Lucero et al. 1998; van Anken et al. 2003). The acidic or neutral pI of RIP-II molecules might impart a functional contribution to their ER localization to permit processing by PDI. In contrast, the basic charge of RIP-I may contribute to Golgi- and ER-independent endocytosis (Vives et al. 2003; Sandvig and van Deurs 2005; Vago et al. 2005).

To establish the role of electrostatic interactions in targeting the ribosome by the RIP, we examined ribosome

cleavage reactions with RIP of both types. The sensitivity of the reaction rate to the salt concentration provides a well-established metric of electrostatic interactions. Accordingly, we acquired salt-rate profiles for RIP-I (saporin and gypsophilin) and RIP-II (ricin A) with ribosomes from rat liver (Fig. 2A). Saporin ($pI_{\text{calc}}=9.5$) and gypsophilin ($pI=10.1$) (Yoshinari et al. 1997) have basic isoelectric points and therefore represent the majority of RIP-I (Fig. 1C). Ricin A, the most studied N-glycosidase to date, has an isoelectric point in the median of pI_{calc} distribution for RIP-II (Fig. 1C).

We determined the rates of N-glycosidase-catalyzed depurination by following production of the ~ 400 nucle-

otide (nt) long α fragment, generated via β elimination within the SRL of 28S rRNA and aniline cleavage of the abasic site (Fig. 2B,C; Materials and Methods). Similar to that of restrictocin, reaction profiles exhibited a strong linear dependence on KCl concentration in double-logarithmic coordinates ($n=\partial \log[k_{\text{cat}}/K_m]/\partial \log[\text{KCl}]$) in the 30–90 mM concentration range, suggesting that electrostatic interactions contribute to ribosome targeting by the three N-glycosidases. The salt dependence was significantly higher for saporin ($pI_{\text{calc}}=9.5$, $n=-5.2$) and gypsophilin ($pI=10.1$, $n=-5.2$) than for ricin A ($pI_{\text{calc}}=7.1$, $n=-2.5$), indicating greater contribution of electrostatics to reactions with more cationic RIP. At ~ 130 mM KCl, the same

FIGURE 2. Sedimentation and RIP cleavage properties of ribosomes isolated from rat liver. (A) Sedimentation profile of rat liver ribosomes determined by analytical ultracentrifugation. (B) Agarose gel electrophoresis analysis of ribosome cleavage by ricin A, saporin, and gypsophilin. (C) Quantitation of the data in B. (D) Salt-rate profiles for cleavage of rat liver ribosomes by ricin A, saporin, and gypsophilin. The data were fit to a salt-dependent Michaelis–Menten equation (Materials and Methods). (E,F) Measurement of kinetic constants k_{cat} and K_m for saporin-catalyzed ribosome cleavage at 50 mM KCl (E) and 100 mM KCl (F).

cooperative stimulatory transition occurred in all three profiles (Fig. 2D; Hill coefficient $m=15 \pm 4$; Materials and Methods), as observed previously for restrictocin (Korennykh et al. 2006). This transition coincides with the appearance of free ribosomal subunits as monitored by analytical ultracentrifugation. At salt concentrations above the transition, the data fit to the pretransition slope (n) for each N-glycosidase, suggesting that the rearrangement in the ribosome does not alter the electrostatic interactions with RIP.

As the salt concentration decreases, the second-order rate constant k_{cat}/K_m increases due to tighter binding of RIP to the ribosome (Fig. 2E,F). Finally, below 30 mM KCl saporin and gypsophilin cleave ribosomes with maximum $k_{\text{cat}}/K_m=(1 \pm 0.2) \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$. This value approaches the encounter frequency predicted from the Smoluchowski equation, which estimates that diffusion-controlled encounters between saporin or gypsophilin and the ribosome occur with a frequency of $(1.8 \pm 0.4) \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ (Materials and Methods). Favorable electrostatic interactions provide the only known mechanism to allow binding at the encounter frequency (von Hippel and Berg 1989; Camacho et al. 1999; Selzer and Schreiber 1999; Selzer et al. 2000; Korennykh et al. 2006). Consistent with this view, the least positively charged RIP ricin A ($\text{pI}_{\text{calc}}=7.1$) exhibits a weaker salt dependence ($n=-2.5$) and depurinates ribosomes with k_{cat}/K_m not exceeding $7 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ (Fig. 2D).

Gypsophilin exhibits the highest second order rate constant among the tested N-glycosidases, at every salt concentration used. We estimate that SRL occupies $\sim 1/600$ of the ribosomal surface area (SRL size $\sim 20 \times 20 \text{ \AA}$; ribosome hydrodynamic radius $R_h \sim 140 \text{ \AA}$; Nieuwenhuysen et al. 1983), suggesting that no more than one out of ~ 600 collisions between gypsophilin and the ribosome occurs with the enzyme active site directly encountering the SRL. In the absence of forces that would steer gypsophilin to the SRL prior to collision or allow long-lived diffusion of the RIP along the ribosomal surface subsequent to collision, k_{cat}/K_m for ribosome depurination should not exceed $1.8 \times 10^{10} \text{ M}^{-1}\text{s}^{-1} / 600$, or $\sim 3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. The experimental k_{cat}/K_m of gypsophilin considerably exceeds this basal value at all salt concentrations tested (Fig. 2D), suggesting that electrostatic interactions facilitate the diffusion of gypsophilin toward the SRL over a wide range of salt concentrations.

Our new data with three different RIP support the view that electrostatics has general importance for RIP function. We found unforeseen parallels in ribosome targeting by RIP of type-I and type-II and α -sarcin endonucleases. All toxins that we have tested catalyze ribosome cleavage with a steep dependence on the salt concentration (n) (Table 1), indicating that they exploit multiple electrostatic interactions with the ribosome. As a consequence, these enzymes can operate on the ribosome with k_{cat}/K_m exceeding their

TABLE 1. Kinetic parameters for cleavage of rat liver ribosomes by ricin A, saporin, gypsophilin, and restrictocin at 50 mM KCl, 1 mM MgCl_2 , 37°C

	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)	n
Ricin A	2600 ^a	29.6 ^a	1.2×10^8 (1.0×10^7) ^a	-2.5
Saporin	55	62	1.5×10^9	-5.2
Gypsophilin	—	—	5.0×10^9	-5.2
Restrictocin ^b	30	1	3×10^7	-6.0

^aEndo and Kunio (1988). Data were determined at 25 mM KCl and 5 mM MgCl_2 .

^bKorennykh et al. (2006).

basal encounter frequency of $\sim 3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ by more than an order of magnitude. Three toxins with basic pI_{calc} , restrictocin, saporin, and gypsophilin, achieve the Smoluchowski limit under low salt conditions, thereby rivaling the speed of the fastest enzyme, superoxide dismutase (SOD) (Folcarelli et al. 1999), except the toxins operate on a far larger and a more complex substrate than does SOD. Notably, restrictocin bears no sequence or structural similarity with the RIP, suggesting that structurally dissimilar proteins may exploit the electrostatics of the ribosome with the same efficiency. In conclusion, Coulomb interactions may have more general roles than previously anticipated in helping proteins to find their ribosomal target sites.

MATERIALS AND METHODS

Bioinformatics

The ExPasy protein server was used to identify 96 nonredundant RIP-I and RIP-II sequences and calculate their isoelectric points (Gasteiger et al. 2003).

Analysis of salt dependence

The salt-rate profiles for the ribosome depurination by ricin A, saporin and gypsophilin (Fig. 2D) were fit to a salt-dependent Michaelis–Menten equation obtained by replacing the single-turnover catalytic constant k_2 in the salt-dependent Michaelis–Menten equation (Korennykh et al. 2006) with a sum

$$k_2 + a \left(\frac{[\text{KCl}]^m}{K_{\text{trans}}^m + [\text{KCl}]^m} \right) \quad (1)$$

where m accounts for the positive cooperative transition occurring at $\sim 130 \text{ mM KCl}$. The variable a is proportional to the amplitude of the transition and measures the sensitivity of a given RIP to the ribosome rearrangement occurring at 130 mM KCl , K_{trans}^m is the midpoint of the transition. The average values of K_{trans}^m and m were 130 mM and 15 , respectively. The value of a was 80 for saporin and 780 for ricin A and gypsophilin.

Encounter rate calculations

The Smoluchowski encounter rate was calculated using Equation (2) (von Hippel and Berg 1989),

$$k = 4\pi N_0 (R_h^{\text{RIP}} + R_h^{\text{Rb}}) (D_h^{\text{RIP}} + D_h^{\text{Rb}}) / 1000 \quad (2)$$

where k is the bimolecular encounter rate constant ($\text{M}^{-1}\text{s}^{-1}$), N_0 is Avogadro's number, R_h values are the hydrodynamic radii of RIP and the ribosome (cm), and D_h values are their diffusion coefficients ($\text{cm}^2 \text{s}^{-1}$). Diffusion coefficients D_h for the 80S ribosome and saporin are $(1.3 \pm 0.3) \times 10^{-7} \text{ cm}^2 \text{s}^{-1}$ (Donceel et al. 1982; Nieuwenhuysen et al. 1983) and $1.33 \times 10^{-6} \text{ cm}^2 \text{s}^{-1}$ (Supplemental Fig. 4), respectively. Hydrodynamic radii R_h are $(140 \pm 30) \times 10^{-6} \text{ cm}$ for the 80S particle (Nieuwenhuysen et al. 1983; Dube et al. 1998) and $24 \times 10^{-6} \text{ cm}$ for saporin (Supplemental Fig. 4). Substitution of the corresponding R_h and D_h values in Equation (2) gives a bimolecular encounter rate constant k of $(1.8 \pm 0.4) \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$.

Biophysical analyses

To ensure that the 80S particles were intact, analytical ultracentrifugation of ribosomes at different salt concentrations was performed on a Beckman XL-A, as described previously (Korennykh et al. 2006). Dynamic light scattering was performed on a PD-2020 dynamic light scattering detector. Experiments were conducted at the Biophysical Core facility of the University of Chicago. Before each run, the detector was calibrated with 2 mg mL^{-1} of bovine serum albumin (Supplemental Fig. 4).

Ribosome cleavage assay

Saporin from *Saponaria officinalis* and ricin A chain from *Ricinus communis* were purchased from Sigma. Gypsophilin from *Gypsophila elegans* was a gift from Y. Endo (Ehime University, Matsuyama, Japan), Y.-L. Chan (University of Chicago), and I.G. Wool (University of Chicago). Ribosome cleavage reactions and data analysis were carried out as described previously for restrictocin (Korennykh et al. 2006). Briefly, reactions were conducted at 37°C and contained 10 mM Tris-HCl (pH 7.4), 0.05% (v/v) Triton X-100, KCl as indicated, 1 mM MgCl_2 , and $2\text{--}10 \text{ nM}$ ribosomes. Before gel analysis, samples of total rRNA were resuspended in $100 \mu\text{L}$ of a $0.7:1$ mixture of aniline and glacial acetic acid (v/v) and incubated at 37°C for 15 min to induce cleavage of rRNA backbone at the abasic site resulting from the N-glycosidase action. After incubation, rRNA was ethanol precipitated, dissolved in 10 M urea loading solution, analyzed by 1.5% (w/v) agarose gel electrophoresis, and visualized by ethidium bromide staining. The cleavage of rRNA was quantified as described previously (Korennykh et al. 2006), by disappearance of the 28S band and by appearance of the α fragment, using the 18S rRNA and 5S+5.8S bands as internal standards. The N-glycosidases examined cleaved 28S rRNA to greater than 90% completion with single exponential kinetics.

Experimental errors

All kinetic values were determined multiple times. Experimental errors are included in the text where appropriate.

SUPPLEMENTAL DATA

All Supplementary Figures and materials can be obtained at http://netcpp.com/2007/RNA/Toxins_Suppl.pdf or by e-mail: avkoren@alumni.uchicago.edu.

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