Steady-State Kinetics of Ricin A-Chain Reaction with the Sarcin−Ricin Loop and with HIV-1 Ψ-RNA Hairpins Evaluated by Direct Infusion Electrospray Ionization Mass Spectrometry

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Direct infusion electrospray ionization mass spectrometry (ESI)[1] offers a viable quantitative approach for the rapid and accurate determination of kinetic parameters for enzymatic reactions involving relatively small substrates/products (up to ∼1 kDa).[2] In this report, the kinetic analysis of large (up to ∼12 kDa) RNA substrates is obtained directly by ESI, with no need for either off-line or on-line separation of products from starting substrates and without the traditional use of radioactive tracers. In contrast with reports describing direct infusion ESI or LC−MS methods for kinetic analysis,[2,3] quantitative data are obtained in a more rapid and direct way by using the molar fractions of products and substrates calculated from the relative ion intensities, with no need for internal standards or calibration curves.

The reaction investigated involves ricin, which is a powerful cytotoxic protein that compromises the ribosomal function in protein synthesis by attacking a universally conserved hairpin in the 28S subunit of eucaryotic rRNA.[4] The catalytic activity of this heterodimeric glycoprotein is afforded by the A-chain, which hydrolyzes the N-glycosidic bond of an essential adenine in the ribosomal elongation site, blocking the association of elongation factors, the binding of aminoacyl-tRNA, and the translocation process. It has been calculated that a single molecule is capable of killing one cell.[5] For this reason, ricin has been investigated for possible use in virus and cancer therapy as the cytotoxic component of immunotoxins prepared by conjugation with antibodies targeting viral or neoplastic antigens.[6]

While ricin has proven active against the ribosomal RNA of HIV-infected cells, it has failed to produce detectable depurination of purified viral RNA.[7] The putative recognition sequence for the toxin includes a double-stranded stem and a GAGA tetranucleotide single-stranded loop (the susceptible adenine is underlined).[8,9] This exact motif is replicated by the stem-loop hairpin SL4 present in the ψ-RNA site of HIV-1 (Figure 1a),[10] which suggests the region as a possible target for ricin. This site, also called the packaging signal, is a highly conserved region of viral genome that is essential to the dimerization and encapsidation of genomic RNA in the final maturation of the virion.[11]

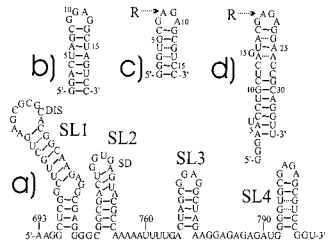

Figure 1. Sequences of the HIV-1 ψ-RNA site (a) and of the model hairpins employed for his study: SL3 (b), SL4 (c), and 35mer hairpin from 28S rRNA (d). The sequences of intact ψ-RNA, SL3, and SL4 are drawn according to ref 11e; the 35mer hairpin was plotted according to ref 9c.

The capability of direct infusion ESI to provide quantitative data for RNA substrates was tested on a 35mer oligonucleotide (Figure 1d) with sequence corresponding to the classic sarcin−ricin loop (SRL) from 28S rRNA.[8] The hydrolysis of adenine was monitored by following the loss of 117.1 Da from the starting hairpin as a function of time. The experimental procedure[12] includes the use of ammonium citrate buffer at pH 5.0 for both reaction medium and ESI analysis. This pH was selected as a compromise between the optimal pH reported for the reaction of ricin with the 35mer SRL (pH 7.6)[8d] and that for smaller stem-loop hairpins (pH 4.0).[13,14] Ammonium citrate was also preferred

(1) (a) Whitehouse, C. M.; Dreyer, R. M.; Yamashita, M.; Fenn, J. B. *Anal. Chem.* **1985**, *57*, 675−679. (b) Smith, R. D.; Loo, J. A.; Ogorzalek-Loo, R. R.; Busman, M.; Udseth, H. R. *Mass Spectrom. Rev.* **1991**, *2*, 359−451.

(2) (a) Lee, E. D.; Mück, W.; Henion, J. D.; Covey, T. R. *J. Am. Chem. Soc.* **1989**, *111*, 4600−4604. (b) Wu, J.; Takayama, S.; Wong C.-H.; Siuzdak, G. *Chem. Biol.* **1997**, *4*, 653−657. (c) Bothner, B.; Chavez, R.; Wei, J.; Strupp, C.; Phung, Q.; Schneemann, A.; Siuzdak, G. *J. Biol. Chem.* **2000**, *275*, 13455−13459.

(3) (a) Hsieh, F. Y. L.; Tong, X.; Wachs, T.; Ganem, B.; Henion, J. *Anal. Biochem.* **1995**, *229*, 20−25. (b) Brown, R. P. A.; Aplin, R. T.; Shofield, C. J. *Biochemistry* **1996**, *35*, 12421−12432.

(4) (a) Olsnes, S.; Pihl, A. In *Molecular Action of Toxins and Viruses*; Cohen, P., van Heyningen, S., Eds.; Elsevier Biomedical Press: New York, 1982; pp 51−105. (b) Lamb, F. I.; Robertus, L. M.; Lord, J. M. *Eur. J. Biochem.* **1985**, *148*, 265−270.

(5) Eiklid, K.; Olsnes, S.; Pihl, A. *Exp. Cell Res.* **1980**, *126*, 321−326.

(6) (a) Pincus, S. H.; Wehrly, K.; Cole, R.; Fang, H.; Lewis, G. K.; McClure, J.; Conley, A. J.; Wahren, B.; Posner, M. R.; Notkins, A. L.; Tilley, S. A.; Pinter, A.; Eiden, L.; Teintze, M.; Dorward, D.; Tolstikov, V. V. *AIDS Res. Hum. Retroviruses* **1996**, *12*, 1041−1051. (b) Pastan, I. *Biochim. Biophys. Acta* **1997**, *1333*, C1−C6. (c) Berger, E. A.; Moss, B.; Pastan, I. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 11511−11513.

(7) Rajamohan, F.; Venkatachalam, T. K.; Irvin, J. D.; Uckun, F. M. *Biochem. Biophys. Res. Commun.* **1999**, *260*, 453−458.

(8) (a) Endo, Y.; Chan, Y. L.; Lin, I.; Tsurugi, K.; Wool, I. G. *J. Biol. Chem.* **1988**, *263*, 7917−7920. (b) Endo, Y.; Tsurugi, K. *J. Biol. Chem.* **1988**, *263*, 8735−8739. (c) Endo, Y.; Glück, A.; Wool, I. G. *J. Mol. Biol.* **1991**, *221*, 193−207. (d) Glück, A.; Endo, Y.; Wool, I. G. *J. Mol. Biol.* **1992**, *226*, 411−424.

(9) (a) Ready, M. P.; Kim, Y.; Robertus, J. D. *Proteins* **1991**, *10*, 270−278. (b) Monzingo, A. F.; Robertus, J. D. *J. Mol. Biol.* **1992**, *227*, 1136−1145. (c) Seggerson, K., Moore, P. B. *RNA* **1998**, *4*, 1203−1215.

(10) (a) Clever, J.; Sassetti, C.; Parslow, T. G. *J. Virol.* **1995**, *69*, 2101−2109. (b) McBride, M. S.; Panganiban, A. T. *J. Virol.* **1996**, *70*, 2963−2973. (c) Clever, J.; Parslow *J. Virol.* **1997**, *71*, 3407−3414. (d) Pappalardo, L.; Kerwood, D. J.; Pelczer, I.; Borer, P. N. *J. Mol. Biol.* **1998**, *282*, 801−818. (e) Damgaard, C. K.; Dyhr-Mikkelsen, H.; Kjems, J. *Nucleic Acids Res.* **1998**, *26*, 3667−3676.

(11) (a) Mervis, R. J.; Ahmad, N.; Lillehoj, E. P.; Raum, M. G.; Salazar, F. H.; Chan, H. W.; Venkatesan, S. *J. Virol.* **1988**, *62*, 3993−4002. (b) Linial, M. L.; Miller, A. D. *Curr. Top. Microbiol. Immunol.* **1990**, *157*, 125−152. (c) Gelderblom, H. R. *AIDS* **1991**, *5*, 617−637. (d) Henderson, L. E.; Bowers, M. A.; Sowder, R. C., II; Serabyn, S. A.; Johnson, D. G.; Bess, J. W., Jr.; Arthur, L. O.; Bryant, D. K.; Fenselau, C. *J. Virol.* **1992**, *66*, 1856−1865. (e) De Guzman, R. N.; Wu, Z. R.; Stalling, C. C.; Pappalardo, L.; Borer, P. N.; Summers, M. F. *Science* **1998**, *279*, 384−388.

(12) Ricin A-chain was purchased from Sigma (St. Louis, MO) and repeatedly washed with 10 mM ammonium citrate (pH 5.0) on a Millipore (Bedford, MA) Ultrafree-0.5 centrifugal filter with 5000 Da MW cutoff. The final protein concentration was determined on the basis of OD_{280} ($\epsilon = 23\,730$ $cm^{-1}\,M^{-1}$). RNA oligonucleotides were produced in-house by using T7 RNA polymerase (Aposhian, H. V.; Kornberg, A. *J. Biol. Chem.* **1962**, *237*, 519−525) provided by Z. R. Wu, HHMI-UMBC, purified by preparative-scale polyacrylamide gel electrophoresis, and buffer-exchanged using the same buffer/ultrafiltration device described above. Final concentrations were determined spectrophotomerically at 260 nm using the appropriate molar absorptivities. Solutions containing 1 μM aliquots of ricin A-chain were incubated in 10 mM ammonium citrate (pH adjusted to 5.0) with different concentrations of hairpin substrates for up to 3 h at 35 °C, while small aliquots were taken at predetermined times and quenched by addition of methanol. Data were obtained in duplicate and fitted to the Michaelis−Menten equation to calculate the reported values and deviations. Electrospray ionization analyses were performed using the first two sectors of a JEOL (Tokyo, Japan) HX110/HX110 four-sector mass spectrometer. Anions were produced by an Analytica of Branford (Branford, CT) thermally assisted electrospray source. A stream of O_2 (U.S.P. grade) was maintained through a coaxial tube over the spray needle to prevent corona discharge. Spectra were the averaged profile of 20−50 linear scans, with a duty cycle of 9−12 s. Resolution was set to 500 (10% valley) by adjusting the slit width; accuracy was 0.04% or better.

(13) (a) Chen, X. Y.; Link, T. M.; Schramm, V. L. *J. Am. Chem. Soc.* **1996**, *118*, 3067−3068. (b) Chen, X. Y.; Link, T. M.; Schramm, V. L. *Biochemistry* **1998**, *37*, 11605−11613.

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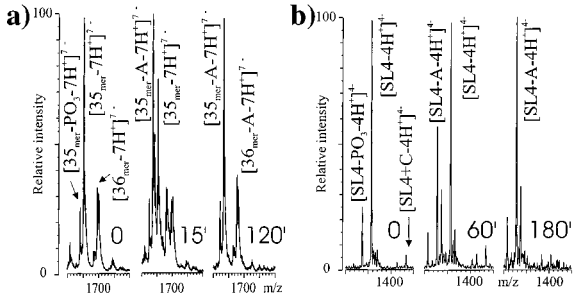

Figure 2. (a) Electrospray mass spectra of 35mer hairpin from 28S rRNA after incubation with ricin A-chain for 0, 15, and 120 min. The region including the $[M - 7H^+]^{7-}$ species is shown. (b) Electrospray mass spectra of SL4 hairpin from HIV-1 ψ-RNA after incubation with ricin A-chain for 0, 60, and 180 min. The region including the $[M - 4H^+]^{4-}$ species is shown.

for its ability to displace and chelate alkali cations, thus reducing the incidence of adducts, which are frequently observed in mass spectrometric analysis of nucleic acids.[15]

The negative ion mode ESI mass spectra of a solution containing ricin A-chain and 35mer hairpin at incubation times of 0, 15, and 120 min are shown in Figure 2a. The major species present in the RNA preparation used as substrate was the 35mer RNA (experimental mass 11 566.4 ± 1.8 Da, calculated from sequence 11 562.1 Da). Minor components were identified as a 34mer failure sequence lacking one U at the 3′-end (exp. −306.3 Da); a 35mer including a nucleotide diphosphate (NDP) at the 5′-end, instead of a triphosphate nucleotide (NTP) (exp. −80.0 Da); and a 36mer including an extra C at the 3′-end (exp. +305.4 Da). These RNA oligonucleotides with slightly different lengths are not resolved by preparative-scale polyacrylamide gel electrophoresis, which shows only sharply defined bands for the polymerase products.[12]

Signals corresponding to depurinated analogues of all the species present in the starting mixture progressively increase during the course of the reaction and are the only ions detected after 120 min. These results confirm that ricin is capable of performing depurination under the low-ionic-strength, no-glycerol conditions that are best suited for direct electrospray analysis.

Concentrations of substrate and product for the kinetic determinations were calculated using the molar fractions obtained directly from the relative ion intensities, knowing the initial substrate concentration. In this particular case, in which a large substrate loses a relatively small and neutral group, a reasonable assumption can be made that substrate and product have comparable ionization efficiency and there is no appreciable detection discrimination between the two. This assumption is supported by the fact that negative charges are carried by phosphate groups in the phosphodiester chain, while the catalytic reaction releases a formally neutral group (adenine base), which does not carry any negative charge. This was confirmed by the full range ESI spectra (not shown), which revealed no appreciable change in the charge state distribution for substrates and products upon adenine hydrolysis. To compare these results with those provided by standard methods, which are not capable of resolving the individual contributions from slightly different components in the substrate mixture, the initial concentration is assumed to correspond solely to the major species 35mer.

Experimental data obtained by reaction of 1 μM ricin A-chain with different initial concentrations of 35mer RNA were used to construct a double-reciprocal plot, which provided $K_m = 19 \pm 3$ μM and $k_{cat} = 0.21 \pm 0.03$ min^{-1}, as compared to13.6 μM and 0.023 min^{-1}, respectively, obtained by Glück et al. at a different pH (pH 7.6) and using radioactive labels and gel electrophoresis.[8d]

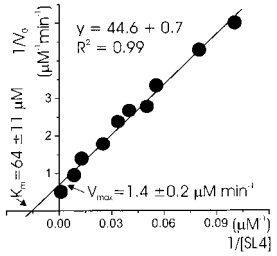

Figure 3. Double-reciprocal plot obtained for the reaction of 1 μM ricin A-chain with different concentrations of HIV-1 SL4 hairpin after 5 min incubation (less than 10% reaction) in 10 mM ammonium citrate buffer (pH adjusted to 5.0).

Close correlation with these previously reported results provided validation to the ESI-based method, which was then applied to the reaction of SL4 from HIV-1 (Figure 1c). ESI spectra at incubation times of 0, 60, and 180 min are shown in Figure 2b. The charge state distribution spans from $[M - 3H^+]^{3-}$ to $[M - 5H^+]^{5-}$ and provides an experimental mass of 5432.3 ± 2.5 Da (5431.1 Da average molecular weight calculated from sequence) for the 16mer SL4. Small percentages of a 17mer hairpin, which includes an extra C (exp. +305.2 Da) at the 3′-end, and of a 16mer, which begins with a diphosphate at the 5′-end (exp. −80.0 Da), are also detected. Loss of adenine from each of these species is readily observed and is complete after 180 min incubation with ricin A-chain. Data obtained with different concentrations of substrate were used to construct a double-reciprocal plot (Figure 3) that provided $K_m = 64 \pm 11$ μM and $k_{cat} = 1.4 \pm 0.2$ min^{-1}.

This is the first experimental evidence that ricin efficiently depurinates in vitro the stem-loop SL4 from HIV-1, in stark contrast with a recent report, which used as a substrate the entire genomic RNA purified from the virus.[7] Denaturation of SL4 by decreasing the pH to 2.0 and then returning it to 5.0, without proper temperature-aided annealing, also caused a loss of activity. This confirms the need for the substrate to possess a well-defined three-dimensional structure, with the adenine base protruding from the loop and accessible to the solvent and the enzyme.[9,13] Hydrolysis by ricin A-chain was also attempted on SL3, the hairpin contiguous to SL4 in the ψ-RNA site. SL3 includes a GGAG motif, which is not a stable tetraloop[10d] (Figure 1b). This reaction showed no evidence of depurination, confirming the specificity of the GAGA recognition sequence for ricin A-chain at 1 μM concentration.

In summary, we have demonstrated that direct infusion ESI mass spectrometry can provide a rapid and quantitative method for detection and kinetics analysis of structure-specific depurination of RNA hairpins by ricin. Viable kinetics data can be obtained for large RNA substrates and potentially for mixtures thereof, with no need for separation of products from starting substrates, nor for internal standards or calibration curves. In addition, in contrast with previous findings,[7] ricin is shown to specifically depurinate the SL4 stem-loop of the HIV-1 ψ-recognition element, suggesting SL4 as a possible target for the proposed development of ricin-based immunotoxins for the treatment of AIDS.[6c]

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(14) In agreement with observations reported by Chen et al.,[13b] preliminary results with the 35mer hairpin could not reproduce the high rates of reaction obtained at neutral pH reported in previous work,[8d] suggesting poor pH control at the low buffer concentrations used in such work. Reaction of SL4 at neutral pH showed no evidence of depurination.

(15) (a) Pieles, U.; Zürcher, W.; Schär, M.; Moser, H. E. *Nucleic Acids Res.* **1991**, *22*, 3191−3196. (b) Murray, K. K. *J. Mass Spectrom.* **1996**, *31*, 1203−1215. (c) Nordhoff, E.; Kirpekar, F.; Roepstorff, P. *Mass Spectrom. Rev.* **1996**, *15*, 67−138.

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