Efficient Modification of RNA by Porphyrin Cation Photochemistry: Monitoring the Folding of Coaxially Stacked RNA Helices in tRNA^{Phe} and the Human Immunodeficiency Virus Type 1 Rev Response Element RNA†

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ABSTRACT: Coaxially stacked RNA helices are a determinant of RNA tertiary structure, but their presence is rarely detected using conventional chemical modification methods. In this report we describe a porphyrin ion photoreaction that enables one to monitor RNA stacking interactions and the folding of coaxially stacked RNA helices. The porphyrin cations *meso*-tetrakis(4-*N*-methylpyridyl)porphine, *meso*-tetrakis-(*para*-*N*-trimethylanilinium)porphine, and *meso*-tetrakis(2-*N*-methylpyridyl)porphine were used to characterize tRNA^{Phe} and the human immunodeficiency virus type-I Rev response element RNA. Nucleosides at the bases of contiguous RNA helices in each RNA are efficiently modified by the porphyrin cations following irradiation of porphyrin−RNA mixtures. These photomodifications are markedly reduced for RNA equilibrated in ionic buffers that lead to enhanced stabilization of coaxially stacked helices. The porphyrin cation photoreaction specifically modifies G18, G20, and G34 in the tRNA folding produced by Mg(II). These nucleobases are exposed to solvent in the native tRNA structure and thus available to stack with solvent-borne porphyrin molecules. The described porphyrin cation photochemical method provides a novel approach to study the solvent accessibility of nucleobases in RNA structure and to monitor the folding of coaxially stacked helices in RNA.

Coaxially stacked RNA helices are comprised of two or more contiguous helices folded into colinear arrays. These extended secondary structure elements are widely found in folded RNA molecules, but their assignments are usually derived from conjecture only after finding related motifs in RNA molecules for which a precise three-dimensional structure is known. Few methods exist that enable one to infer the presence of coaxially stacked helices in folded RNA. For example, the method of comparative sequence analysis allows one to infer coaxial stacking in a functionally related set of molecules if one helix in a group of organisms is longer while its coordinating helix in that group is shorter by the same amount, thereby preserving the overall combined length of the extended helical array (Woese et al., 1983). The technique of helix fusion represents one experimental approach that provides information about coaxially stacked RNA helices. In this approach, two contiguous helices are held together by a phosphodiester bond linker. An RNA molecule is assumed to possess coaxially stacked helices if it adopts a conformation similar to the fused RNA molecule (Murphy et al., 1994). These approaches can be used to identify coaxially stacked helical domains, yet no solution method is available that offers dynamic information about these structural motifs in folded RNA molecules.[1] Reliable solution methods are necessary to detect the presence of coaxial stacking in folded RNA and to monitor their formation during folding transitions in higher order RNA structure.

Among the established solution chemistries that are available for studying nucleic acid conformation, porphyrins comprise a structurally diverse class of complex conjugated aromatic ring systems that can be applied to define complementary shape-selective pockets within folded nucleic acid molecules (Dabrowiak et al., 1989; Fiel, 1989; Lu et al., 1990; Wilson et al., 1993; Nussbaum et al., 1994).[2] Cationic porphyrin molecules possess positive charge character and novel structural properties, making these molecules an unusual group of reagents useful for defining conformational states of nucleic acids. These molecules have been used extensively to characterize DNA structure (Banville et al., 1986; Sari et al., 1990; Munson & Fiel, 1992; Croke et al., 1993; Nussbaum et al., 1994) but have not been widely applied as reagents to analyze RNA structure. Foster and co-workers have studied the interactions of yeast tRNA^{Phe} with T4MPyP[3] by UV−visible spectroscopy, circular dichroism, and thermal denaturation (Foster et al., 1988) and by nuclear magnetic resonance spectroscopy (Birdsall et al., 1989). They proposed that a specific binding site exists in tRNA that allows this compound to bind with high specific-

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[1] Coaxial stacking of contiguous helices has been detected using crystallographic diffraction methods (Kim et al., 1974; Robertus et al., 1974), nuclear magnetic resonance spectroscopy (Puglisi et al., 1990; Chastain & Tinoco, 1992), and electrophoretic mobility assay (Duckett et al., 1995).

[2] Metal-liganded porphyrins have been used extensively to probe nucleic acid structure (Pasternack et al., 1983; Ward et al., 1986; Bütje & Nakamoto, 1990; Ding et al., 1990; Meunier, 1992).

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ity. To better characterize this porphyrin−RNA interaction, we have applied T4MPyP and the structurally related TMAP and T2MPyP porphyrin cations as reagents to probe the native conformation of tRNA. We found that these porphyrin cations efficiently promote conformation specific modification of nucleobases located in the hinge regions of tRNA. The RNA helices of these hinge regions coaxially stack in the native tRNA tertiary structure. We have also applied the porphyrin ion photochemical method to study the folded conformation of the Rev responsive element RNA of the human immunodeficiency virus type-1. Our modification studies provide additional support for one proposed coaxial stacking interaction within the folded structure of the HIV-1 RRE RNA. This porphyrin-mediated photoreaction of RNA may have novel applications in other systems for monitoring RNA folding domains with the propensity to form coaxially stacked RNA helices in higher order structure.

MATERIALS AND METHODS

Materials. All buffer solutions were prepared with sterile water that was initially deionized using a Millipore MilliQ Plus water purification system. All buffer components that were used in this work were of the highest grade obtainable from various manufacturers. Calf intestinal alkaline phosphatase was obtained from United States Biochemicals; T_4 polynucleotide kinase, *Bst*N1, *Eco*RI, *Sal*I, and *Xho*I were obtained from New England Biolabs; Taq DNA polymerase was obtained from Promega. Bacteriophage T7 RNA polymerase was purified following overexpression of the bacteriophage T7 *gene 1* from BL21/pAR1219 (Davanloo et al., 1984). The [γ-^{32}P]ATP (6000Ci/mmol) was obtained from DuPont-New England Nuclear. The chloride forms of cationic porphyrins *meso*-tetrakis(4-*N*-methylpyridyl)porphine (T4MPyP), *meso*-tetrakis(*para*-*N*-trimethylanilinium)-porphine (TMAP), and *meso*-tetrakis(2-*N*-methylpyridyl)-porphine (T2MPyP) were obtained from Midcentury Chemicals (Posen, IL). The porphyrins were dissolved in sterile water just before use and the extinction coefficients used for calculating concentrations were $\epsilon_{424} = 2.26 \times 10^5$ M^{-1} cm^{-1} for T4MPyP (Pasternack et al., 1983), $\epsilon_{414} = 1.82 \times 10^5$ M^{-1} cm^{-1} for T2MPyP (Pasternack et al., 1983), and $\epsilon_{412} = 4.16 \times 10^5$ M^{-1} cm^{-1} for TMAP (Krishnamurthy, 1977).

Description and Construction of Plasmid Transcription Templates. The plasmid p67YF0 served as the transcription template for *in vitro* synthesis of the mature tRNA^{Phe} RNA molecule (Sampson & Uhlenbeck, 1988) and was kindly provided by O. C. Uhlenbeck. The RRE DNA bounded by coordinate positions 7337 and 7598 of the HIV-1 RNA genome (cf. HIV-1 strain H9Pv.22; Muesing *et al.*, 1985) was amplified from pIIIenvΔKpnI (kindly provided by J. G. Sodroski) using *Taq* DNA polymerase and the following synthetic DNA primers: 5′-GCATGTCGACAGTGGGAATAGGAGCTTTGTTC-3′ and 5′-GCATCTCGAGCAACCCCAAATCCCCAGGAG-3′. These primers encode unique *Sal*I and *Xho*I sites so that the resulting ∼280 bp amplification product could be subcloned into pBRX322 following restriction of the plasmid and DNA amplification product with these enzymes. The plasmid pBRX322 is a derivative of pBR322 that contains an *Xho*I site inserted into the *Eco*RI site. One plasmid subclone that contained a single RRE insert bounded by *Sal*I and *Eco*RI restriction endonuclease sites was cleaved with these enzymes, and the liberated RRE fragment was subcloned into pGEM4 to yield pGEM4RRE. The complete nucleotide sequence of the RRE DNA insert in pGEM4RRE was verified by sequence analysis. This plasmid served as the transcription template for *in vitro* synthesis of the HIV-1 RRE RNA.

Preparation of RNA. The synthetic yeast tRNA^{Phe} was transcribed by T7 RNA polymerase from a *Bst*N1 linearized p67YF0 DNA template using published procedures (Sampson & Uhlenbeck, 1988). The synthetic RRE RNA was transcribed by T7 RNA polymerase from an *Eco*RI linearized pGEM4RRE DNA template. The purified RNA molecules were dephosphorylated by calf intestinal alkaline phosphatase and 5′-^{32}P-end-labeled by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (6000 Ci/mmol) as described by Latham et al. (1990). The radiolabeled RNA products were purified by electrophoresis on a denaturing gel (10% polyacrylamide [acrylamide/bisacrylamide (19:1)]−8 M urea−1× TBE [1× TBE: 0.1 M Tris-HCl (pH 8.3), 0.1 M boric acid, 0.002 M Na_2EDTA]) and recovered from excised gel slices by crushing and soaking in 0.25 M NaCl−0.01 M Tris-HCl (pH 8.0)−0.001 M Na_2EDTA at 4 °C for 4−8 h. The supernatant was recovered from the gel matrix by centrifugation, and the end-labeled RNA molecules were precipitated with ethanol. The RNA molecules were rehydrated in sterile water and refolded by heating the RNA in sterile water for 3 min at 90 °C, followed by the addition of a concentrated stock solution of buffer components during cooling. The composition and concentration of the buffer components that were used in experiments are indicated in the figure legends.

RNA Strand Modification Induced by Porphyrin Cation Photochemistry. The RNA molecules were refolded as described above, and then the porphyrins were added to a final concentration of 5×10^{-8} M. The exposure of the photolabile porphyrin solutions to light was minimized by storing the solutions in amber microfuge tubes and by adding the porphyrins to the RNA solutions under reduced light. Typical reaction conditions consisted of unlabeled RNA (1×10^{-7} M) and [5′-^{32}P]RNA (1×10^{-9} M) in 2×10^{-2} M Tris-HCl (pH 7.5), 1×10^{-4} M EDTA, and different concentrations of $MgCl_2$ and KCl as indicated in the figure legends. Following the addition of the porphyrin to the RNA solutions, the mixtures were equilibrated in the dark on ice for 30 min. Dark reaction control tubes were maintained on ice; photoreaction tubes were inclined on their sides on a bed of ice water and irradiated with 22 mW/cm^2 of visible light ($\lambda > 300$ nm) at 5 cm for 10 min. The RNA samples were precipitated twice from ethanol, rinsed once with 70% ethanol, and dried in a SpeedVac SC100 (Savant). The RNA samples were treated with 1 M aniline acetate (pH 4.6) at 60 °C for 20 min to induce strand scission at the sites of porphyrin cation photomodification (Peattie, 1979). The RNA samples were resuspended in loading buffer [0.010 M Na_2EDTA (pH 8.0), 4 M urea−0.5× TBE, 0.01% (w/v) bromophenol blue, 0.01% (w/v) xylene cyanole] before electrophoresis.

RNA Strand Scission Catalyzed by Fe(II)−EDTA. Iron(II)−EDTA cleavage reactions were performed for the RNA

[3] Abbreviations: T4MPyP, *meso*-tetrakis-(4-*N*-methylpyridyl)porphine; TMAP, *meso*-tetrakis(*para*-*N*-trimethylanilinium)porphine; T2MPyP, *meso*-tetrakis-(2-*N*-methylpyridyl)porphine; Fe(II)−EDTA, iron(II)-ethylenediaminetetraacetic acid; HIV-1, human immunodeficiency virus type-1; RRE, Rev responsive element.

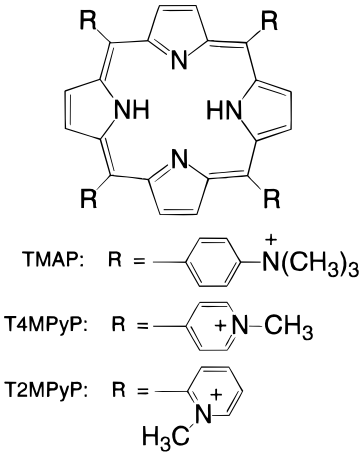

FIGURE 1: Structure of the porphyrin cation compounds used in this study.

molecule using a modification of the reaction described by Latham and Cech (1989). The RNA was refolded in 7 μL of sterile water containing the appropriate salts and buffering components and then 1 μL of 0.010 M $(NH_4)_2Fe(II)(SO_4)_2$−0.020 M Na_2EDTA (pH 8.0) and 2 μL of 0.050 M dithiothreitol were added. The reaction mixtures were incubated on ice for 2 h. Cleavage reactions were quenched with the addition of 1 μL of 0.10 M thiourea. An equal volume (11 μL) of 2× loading buffer [0.020 M Na_2EDTA (pH 8.0), 8 M urea−1.0× TBE, 0.02% (w/v) bromophenol blue, 0.02% (w/v) xylene cyanole] was added prior to electrophoresis.

Sequencing Standards and Gel Electrophoresis Conditions. Sequencing ladders served as marker standards for the RNA structure−mapping experiments. The RNA sequencing ladders of $tRNA^{Phe}$ were generated by limited hydrolysis with RNase T1 and alkali according to the method of Donis-Keller et al. (1977). The end-labeled RNA products were fractionated by electrophoresis on denaturing gels [10% polyacrylamide (acrylamide/bisacrylamide (19:1))−8 M urea−1× TBE]. Following electrophoresis, the gels were dried and exposed to film.

RESULTS

Photomodification of $tRNA^{Phe}$ by Porphyrin Cations. The porphyrin molecules T4MPyP, TMAP, and T2MPyP were used to explore their structure−reactivity profiles with yeast $tRNA^{Phe}$, an RNA molecule whose three-dimensional structure is well characterized (Kim et al., 1974; Robertus et al., 1974; Patel et al., 1987; Figure 1). Irradiation of porphyrin−RNA mixtures with long wavelength light resulted in photodamage at a limited number of sites for $tRNA^{Phe}$ equilibrated in solutions containing Tris buffer and EDTA (Figure 2A). The resulting modifications to the RNA were not frank strand scissions (not shown); hot aniline acetate treatment was required to resolve a site of modification to a strand break. Aniline-sensitive strand breaks were dependent upon both irradiation with long wavelength light and the presence of the porphyrins in the solution. The major site of photomodification in $tRNA^{Phe}$ by all three porphyrin molecules is G65. Additional sites of modification by T4MPyP and TMAP arise near hinge regions and include U12, U16, C27, G43, G45, G46, G51, G53, G57, and C63. T2MPyP modified a subset of the sites (U12, U16, G45, G46, and C63) modified by T4MPyP and TMAP. The reactivity

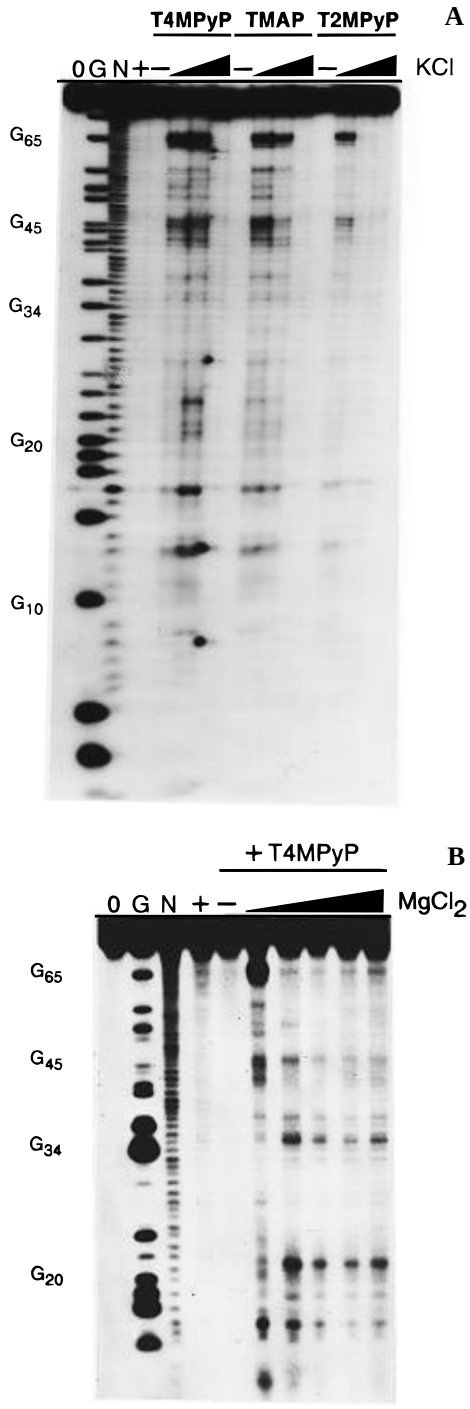

FIGURE 2: Photomodification of $tRNA^{Phe}$ by porphyrin cations. The lanes denoted by 0 represent starting material. The lanes denoted by G and N represent sequencing ladders specific for guanosine and any nucleotide, respectively. The lanes containing irradiated RNA incubated in Tris/EDTA buffer (+) are indicated. The lanes containing nonirradiated porphyrin cation−RNA mixtures incubated in Tris/EDTA buffer (−) are indicated. In panel A, the lanes that contain irradiated porphyrin cation−RNA mixtures are incubated in Tris/EDTA buffers with increasing concentrations of KCl (0, 0.1, and 1 M) as indicated by the gradient symbol. In panel B, the lanes that contain irradiated porphyrin cation-RNA mixtures incubated in Tris/EDTA buffers with increasing concentrations of $MgCl_2$ (0, 5×10^{-4}, 1×10^{-3}, 2×10^{-3}, and 5×10^{-3} M) are also indicated by the gradient symbol. Note that the assignment of product identity in aniline acetate hydrolysis (i.e., relevant to all lanes except those denoted by 0, G, and N) is shifted one nucleotide 3′ to the site of modification relative to the alkaline hydrolysis product since the strand scission promoted by aniline acetate is accompanied by the formation of a protonated Schiff base.

of T2MPyP toward $tRNA^{Phe}$ is also reduced relative to that observed with the two other porphyrin molecules (Figure 2A).

High concentrations of monovalent cations suppress the interaction of porphyrin molecules with DNA and reduce photochemical damage at sites of porphyrin binding (Fiel, 1989; Nussbaum et al., 1994). To explore whether monovalent salts similarly reduce the porphyrin-sensitized photomodification reaction of tRNA, we examined the photochemical reactivity of each porphyrin molecule toward $tRNA^{Phe}$ as a function of KCl concentration. For RNA equilibrated in buffers containing 0.10 M KCl, the porphyrin molecules differed in their reactivity profile with $tRNA^{Phe}$ (Figure 2A). In addition to those sites of modification observed for the RNA equilibrated in buffers lacking KCl, T4MPyP-sensitized photomodification of G22 was also observed in the presence of 0.10 M KCl. Modification was reduced at all TMAP-sensitized sites in buffers containing 0.10 M KCl with the exception of residues U12, U16, and G65. The reactivity of T2MPyP toward $tRNA^{Phe}$ equilibrated with 0.10 M KCl was not detected above the aniline acetate treated, dark control reaction (Figure 2A). For $tRNA^{Phe}$ equilibrated in 1 M KCl, the reactivity of all three porphyrin molecules toward the RNA molecule was diminished considerably relative to their respective reactivity profiles with the RNA equilibrated in buffers lacking KCl (Figure 2A). The minor modification of U12 by T4MPyP persists and a new minor modification of U7 becomes evident. These experiments demonstrate that these porphyrins specifically promote photodamage at select nucleobases in $tRNA^{Phe}$ and that the modification of tRNA displays sensitivity to different concentrations of the K(I) ion.

Divalent cations bind to specific regions within the native structure of $tRNA^{Phe}$ (Danchin, 1972; Kayne & Cohn, 1972; Jack et al., 1977; Holbrook et al., 1977; Quigley et al., 1978) and provide one means whereby the RNA tertiary structure is stably maintained. Birdsall et al. (1989) found that the T4MPyP porphyrin molecule binds in the L-hinge region of the tRNA folding produced by Mg(II); therefore, we investigated whether this site could be detected using the described photochemical method. For RNA equilibrated in buffers containing 0.5 mM $MgCl_2$, the modification of G65 and other sites by T4MPyP is markedly reduced (Figure 2B). Modification at G18, G20, and G34 is enhanced by T4MPyP in reaction buffers containing $MgCl_2$. These marked changes in the reactivity profile of the porphyrin molecule toward Mg(II)-stabilized $tRNA^{Phe}$ are evident for RNA equilibrated in buffers containing concentrations of $MgCl_2$ ranging from 0.5 to 5 mM (Figure 2B).

Protection of Helical Bases to Porphyrin Ion Photomodification Correlates with RNA Tertiary Structure Formation. The tertiary structure of $tRNA^{Phe}$ has been studied with the solvent-based cleavage reagent Fe(II)−EDTA (Latham & Cech, 1989). This reagent generates free radicals in solution that promote oxidative damage of the ribose moieties in both DNA and RNA (Hertzberg & Dervan, 1984; Tullius & Dombroski, 1985, 1986; Burkhoff & Tullius, 1987; Tullius et al., 1987). The resulting strand scission occurs at the site of the oxidized sugar with little or no specificity for primary sequence or secondary structure (Celander & Cech, 1990). Latham and Cech (1989) found that the $tRNA^{Phe}$ tertiary structure displayed regions of protection as well as areas of uniform cleavage by this reagent. The accessibility of ribose sugars to solvent is a major determinant of the extent of strand scission by Fe(II)−EDTA. We performed an Fe(II)−EDTA cleavage assay for $tRNA^{Phe}$ equilibrated in buffers containing either 5 mM $MgCl_2$ or 1 M KCl to assess whether the reactivity of each porphyrin molecule reflected its ability to recognize a specific conformation of $tRNA^{Phe}$ adopted under these buffer conditions. Figure 3 shows that the ribose moieties at positions 18−20 of the D loop, 46−49 of the variable loop, and 57−60 of the T loop of $tRNA^{Phe}$ are protected from free radical mediated damage for RNA equilibrated in buffers containing 5 mM $MgCl_2$ but not in Tris buffers lacking additional salts. These results are similar to the findings reported by Latham and Cech (1989) for Fe(II)−EDTA cleavage of this RNA molecule equilibrated in 10 mM $MgCl_2$ at 42 °C. These ribose moieties are also protected for $tRNA^{Phe}$ equilibrated in buffers containing 1 M KCl (Figures 3 and 4); therefore, we conclude that K(I) stabilizes $tRNA^{Phe}$ in a native-like conformation.[4]

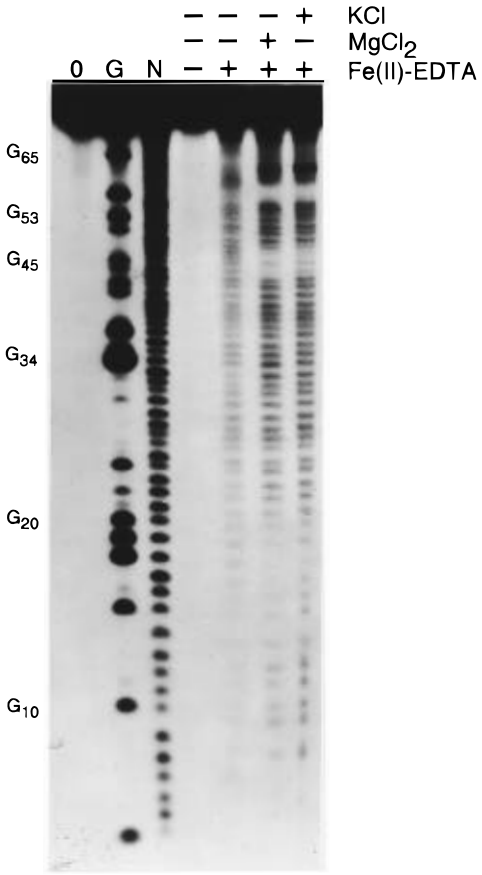

FIGURE 3: Fe(II)−EDTA strand scission of $tRNA^{Phe}$. The lanes denoted by 0, G, and N are described as in the legend to Figure 2. The lanes that contain RNA incubated with either Tris/EDTA buffer, Tris/EDTA buffer containing Fe(II)−EDTA, Tris/EDTA buffer containing 5×10^{-3} M $MgCl_2$ and Fe(II)−EDTA, or Tris/EDTA buffer containing 1 M KCl and Fe(II)−EDTA are shown. Note that the assignment of product identity in Fe(II)−EDTA cleavage (i.e., relevant to all lanes except those denoted by 0, G, and N) is shifted one nucleotide 5′ of the actual site of cleavage relative to the alkaline hydrolysis product since the strand scission promoted by Fe(II)−EDTA is accompanied by the elimination of the ribose moiety.

Detection of Stacked Helical Regions in the HIV-1 RRE RNA by Porphyrin Ion Photochemistry. To determine whether helical stacking interactions that have been proposed for another RNA molecule could be monitored by the described porphyrin cation photochemistry, we used the

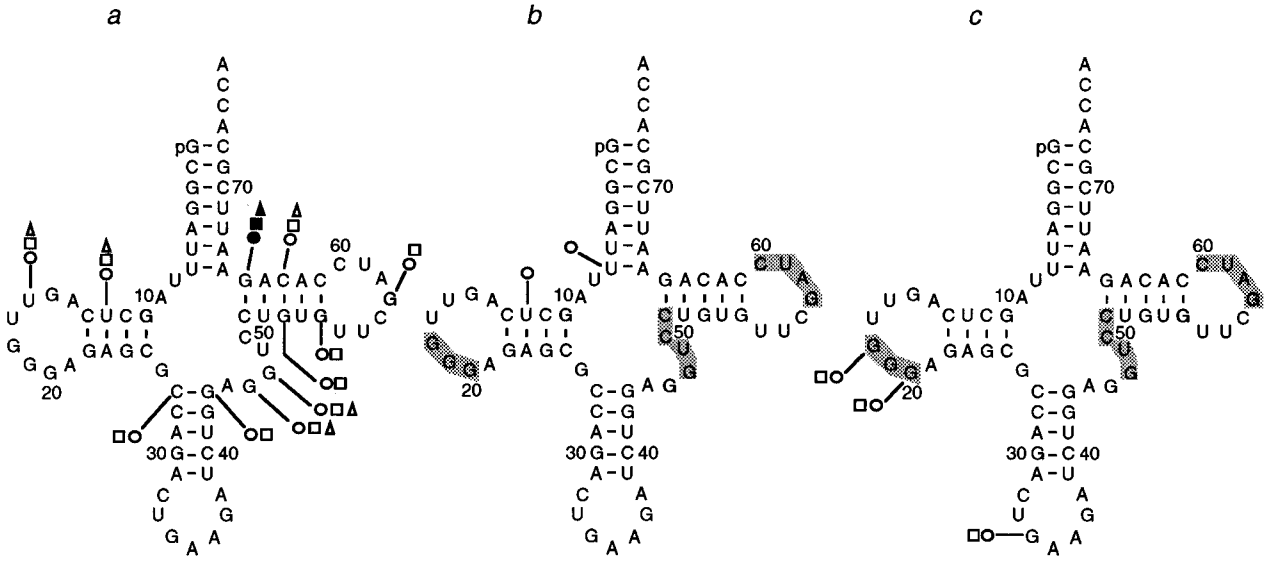

FIGURE 4: Summary of $tRNA^{Phe}$ modification by porphyrin cation photochemistry. The T4MPyP, TMAP, and T2MPyP modifications are superimposed on the secondary structure model of the RNA molecule. The sites of weak (○, □, △) and strong (●, ■, ▲) modification by the T4MPyP (●), TMAP (■), and T2MPyP (▲) are indicated for $tRNA^{Phe}$ equilibrated in Tris/EDTA buffer (a), Tris/EDTA buffer containing 1 M KCl (b), and Tris/EDTA buffer containing 5×10^{-3} M $MgCl_2$ (c). The shaded areas illustrated on the $tRNA^{Phe}$ structures in panels b and c represent RNA regions that are protected from cleavage by the Fe(II)−EDTA reagent for RNA equilibrated under the indicated buffer conditions.

HIV-1 RRE RNA molecule because it possesses an extensive secondary structure (Kjems et al., 1991; Chin, 1992; Mann et al., 1994; Figure 5A). This RNA secondary structure interacts with the viral Rev protein to effect efficient nucleocytoplasmic export of virion RNA and singly-spliced env mRNA (Cullen, 1992). The HIV-1 Rev protein interacts with a single high affinity binding site in stem IIB of the RRE structure at nanomolar concentrations of Rev protein (Heaphy et al., 1991; Karn et al., 1991; Iwai et al., 1992; Tiley et al., 1992). Nuclease protection experiments and electron microscopy studies of Rev protein−RRE RNA complexes have been used to demonstrate that the Rev protein binds to double-stranded portions of the HIV-1 RRE secondary structure at micromolar concentrations of protein (Karn et al., 1991; Heaphy et al., 1991; Mann et al., 1994). The optimal Rev protein binding site is comprised of a coaxially stacked RNA secondary structure comprised of stems I, IIa, and IIb (Mann et al., 1994; Figure 5A). All three porphyrin compounds promote modification of the HIV-1 RRE RNA for RNA equilibrated in buffers containing Tris-HCl and EDTA (Figure 5B). Nucleobases located near or at the ends of helical regions are preferentially modified by T4MPyP and TMAP. The porphyrin cations strongly modified G7385 and weakly modified G7452 and G7510; these particular guanosines lie at the bases of helix IIa, helical region III+IV, and helix V, respectively (Figure 5A). Other guanosines that are weakly modified by these porphyrin cations are located in helical regions that contain internal loops (G7422, G7470, G7472, G7473, G7538, and G7551), noncanonical base pairing interactions (G7393 and G7581), and bulged nucleotides (G7545 and G7546). The porphyrin compound T2MPyP weakly modifies a subset of the sites modified by T4MPyP and TMAP (G7385, G7452, G7470, G7510, G7545, G7546, G7551, and G7581; Figure 5A), which reflects a similar reactivity trend observed with modification of the $tRNA^{Phe}$ molecule by these compounds. One exception to this reactivity profile is seen with the HIV-1 RRE RNA molecule where G7392 is more strongly modified by T2MPyP than by the other two porphyrin compounds (Figure 5B).

Photomodification of many of the aforementioned guanosine residues by the porphyrin cation reagents are reduced for RRE RNA preparations equilibrated in buffers containing 1 M KCl (Figure 5B). Sustained or new photomodifications occur at guanosines located in loops (G7524), at the ends of stems in helix region III+IV (G7452), and in helices I (G7581) and V (G7510, G7527, and G7538), and within a region that contains a bulged nucleotide (G7545 and G7546). Figure 5A illustrates a summary of these modification profiles on the secondary structure of the HIV-1 RRE RNA molecule.

DISCUSSION

A novel finding in this work was that porphyrin cations modify RNA in a conformation specific manner. All three porphyrins selectively modify nucleobases located predominantly at hinge regions that link contiguous RNA helices (Figures 4A and 5A). These hinge regions form coaxially stacked helices in the native tertiary structure of tRNA (Kim et al., 1974; Robertus et al., 1974). The absence of stabilizing counterions renders a tRNA structure considerably more flexible and prone to thermal denaturation (Fresco et al., 1966). Under these more structurally dynamic conditions, the coaxially stacked RNA helices of tRNA are destabilized and allow the porphyrin cation molecules to bind at these hinge regions. A major site of photomodification in tRNA, namely G65, may be attributed to porphyrin−tRNA contacts being stabilized by hydrophobic interactions between

[4] (a) The ribose sugars of the K(I)-stabilized tRNA structure possess the same reactivity toward Fe(II)−EDTA as that displayed by the ribose sugars of the Mg(II)-stabilized tRNA structure; therefore, the sugar−phosphate backbone of the K(I)-stabilized RNA adopts a fold that is similar to that observed for the native structure. The orientation of the nucleobases in tRNA is not monitored by Fe(II)−EDTA. (b) The synthetic $tRNA^{Phe}$ transcript adopts the correct, biologically active conformation, as assessed by aminoacylation assays (Sampson & Uhlenbeck, 1988; Sampson et al., 1989).

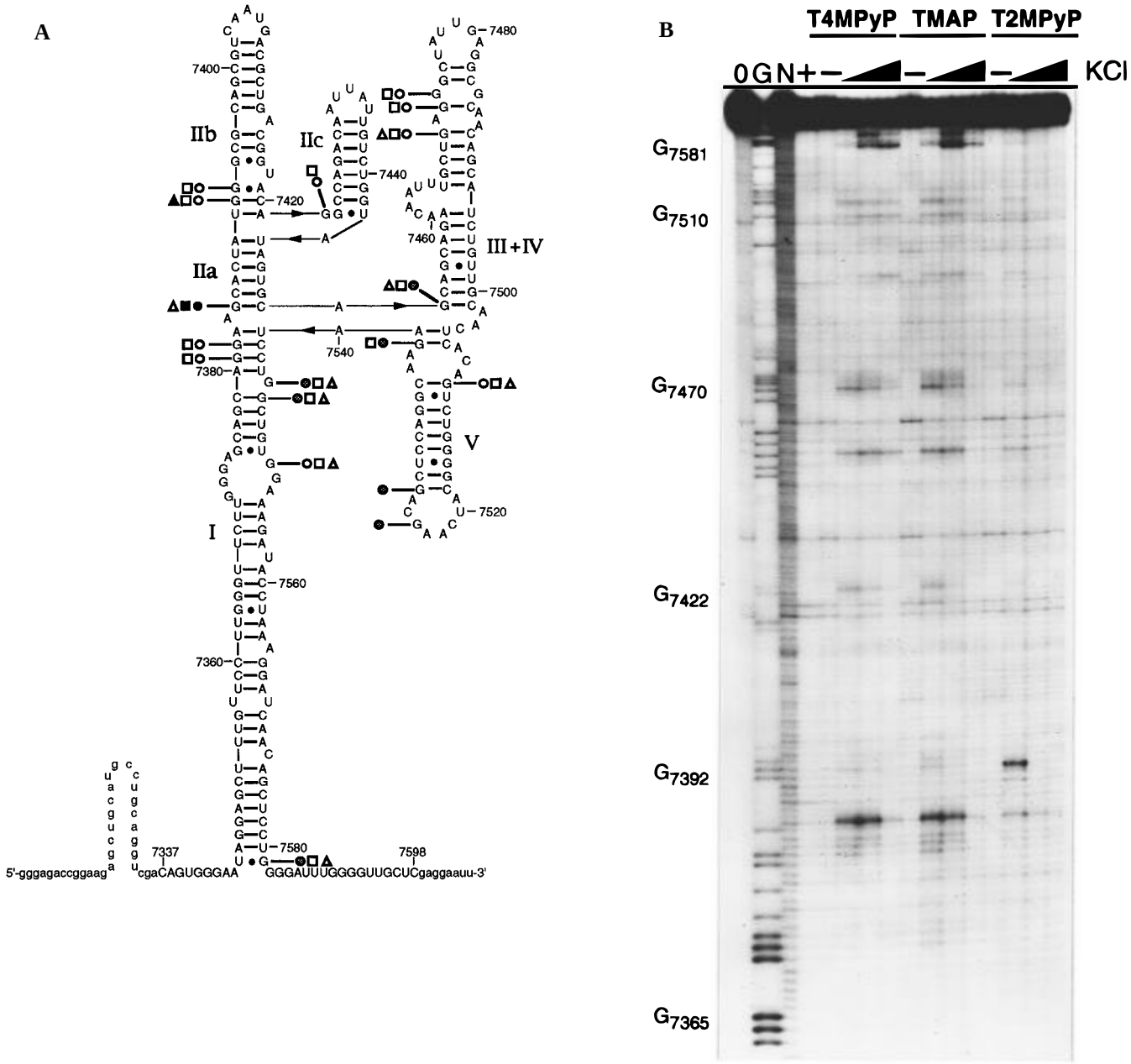

FIGURE 5: Accessible guanosines in HIV-1 RRE RNA are modified by porphyrin ion photochemistry. In panel A, the HIV-1 RRE RNA secondary structure is shown as adapted from Mann et al. (1994); the numbering of the HIV-1 RRE is taken from the HIV-1 RNA genome (strain H9Pv.22) as reported by Muesing et al. (1985). The HIV-1 RRE RNA sequence is illustrated in uppercase letters, while the nucleotide sequence that derives from transcribed vector is shown in lowercase letters. The sites of T4MPyP, TMAP, and T2MPyP modification are superimposed on the secondary structure model of the RNA molecule. The sites of weak (○, □, △) and strong (●, ■, ▲) modification by the T4MPyP (●), TMAP (■), and T2MPyP (▲) are indicated for the HIV-1 RRE RNA equilibrated in Tris/EDTA buffer. The sites of new or sustained modification by T4MPyP (stippled circles) are indicated for the HIV-1 RRE RNA equilibrated in Tris/EDTA buffers containing 1 M KCl. In panel B, the lanes denoted by 0, G, N, (+), and (−) are defined as in the legend to Figure 2. The lanes that contain irradiated porphyrin cation−RNA mixtures are incubated in Tris/EDTA buffers with increasing concentrations of KCl (0, 0.1, and 1 M) as indicated by the gradient symbol.

the planar surfaces of the porphyrin molecule and aromatic ring system of basepairs G65:C49 and/or A66:U7 that reside at the bases of stems in the T-stem/acceptor stem hinge region. Porphyrin−tRNA complexes may be additionally stabilized by cation−π interactions that occur between the positively charged amine cation of the studied porphyrin compounds and the nucleobases of tRNA (Dougherty, 1996). All three porphyrin molecules display limited reactivity with tRNA equilibrated in buffers that promote formation of a native-like RNA conformation; therefore, some aspect of RNA tertiary structure prevents the porphyrin molecules from interacting with the hinge regions in a manner that favors efficient photomodification of nucleobases.

The native conformation of tRNA is stabilized by a structural neighborhood consisting of the variable loop, T loop, and D loop (Figure 4B). These loops participate in the formation of base triples (Levitt, 1969; Kim et al., 1974; Robertus et al., 1974) and interact with divalent cations (Danchin, 1972; Kayne & Cohn 1972; Jack et al., 1977; Holbrook et al., 1977; Quigley et al., 1978). Potassium(I) promotes the formation of a native-like conformation for tRNA, as assessed by the Fe(II)−EDTA cleavage studies;

thus, the divalent cation binding sites that are created in the tertiary structure need not be occupied by divalent cations for acquisition of the native structure. This observation is consistent with previous studies of Fresco and co-workers where they found that molar concentrations of monovalent ions could substitute for millimolar concentrations of divalent ions in stabilizing tRNA tertiary structure (Fresco et al., 1966). The porphyrin cation photoreaction revealed additional insights about the structural features in this region of tRNA that are not evident with the use of the Fe(II)−EDTA cleavage reagent. The T4MPyP porphyrin promoted new modifications in the vicinity where the T loop and D loop approach one another and in the U-turn region of the anticodon loop for tRNA equilibrated in only buffers containing $MgCl_2$. The nucleobases that are modified by T4MPyP (G18, G20, and G34) are highly accessible to solvent in the native structure. These findings are in agreement with those reported by Foster and her co-workers who found that T4MPyP binds to the native tRNA in the vicinity of G18 (Birdsall et al., 1989). We interpret these modifications to stem from stacking interactions that arise between porphyrin molecules and the affected guanosines. The combination of hydrophobic interactions and cation−π interactions may explain why the mode of porphyrin binding to the tertiary structure of tRNA is neither intercalative nor simply electrostatic (Birdsall et al., 1989). The porphyrin compounds therefore provide another means for obtaining additional structural information about accessible nucleobases in the folded RNA structure.

The HIV-1 RRE RNA molecule contains several helical elements. Extensive coaxial stacking interactions have been proposed to form within stem IIb (Battiste et al., 1994, 1995) as well as between helical stems IIb, IIa, and I upon binding of the Rev protein (Mann et al., 1994). Our modification studies support coaxial stacking within stem IIb, and between stems IIa and I; however, we cannot infer from our studies whether coaxial stacking occurs between stems IIa and IIb due to the A:U pairing located at the base of each stem which cannot be modified by the porphyrin cation photoreactions. Guanosine 7422, located adjacent to stem IIc, is protected from porphyrin modification in high ionic strength buffers. Coaxial stacking interactions are favored for helices that possess a G:A mismatch at the helix−helix interface (Walter et al., 1994). A similar G:A mismatch can form with G7422 and A7444 that flank stem IIc (Figure 5A); thus, stem IIc can be extended by one G:A mismatch pairing and allow the stem to coaxially stack with either stem IIa or IIb. Helical regions III+IV and V remain accessible to porphyrin binding since strong modification of nucleosides at the bases of these helices persists using the T4MPyP porphyrin cation as a probe; therefore, it is unclear whether these latter helical elements are involved in any stacking interactions. The stems IIc, III+IV, and V remain accessible to nucleolytic cleavage even in the presence of Rev protein (Mann et al., 1994), which suggests that extensive Rev binding to the RRE RNA target forms along a contiguous double-stranded region comprised of stems IIb, IIa, and I. If stem IIc coaxially stacks on either stem IIb or stem IIa in the free HIV-1 RRE, then Rev binding to the RRE may induce a conformational change in the RNA, resulting in a switch in coaxial stacking partners.

The photomodification of RNA by porphyrin cations reveals novel features of RNA structure that are not discernible with other previously described solution methodologies. In this regard, the photoreaction studies reported here with RNA bear striking similarities to the corresponding photoreactions performed with DNA oligonucleotides that form branched, three-way junction complexes (Nussbaum et al., 1994). Leontis and co-workers postulated that porphyrins bind to a three-way junction DNA complex wherein hydrophobic contacts between planar porphyrin surfaces and the DNA bases flanking the junction are maximized (Nussbaum et al., 1994). These DNA complexes were found to present stronger binding sites for the T4MPyP and TMAP porphyrins than are found in duplex DNA, as assessed by direct binding assays and by photochemical studies performed under different ionic strength conditions. Although T2MPyP elicited greater photochemical modification of RNA than was observed for the studied DNA oligonucleotide complexes, the porphyrin compounds displayed a similar trend of photochemical reactivity for both RNA and three-way junction DNA complexes (T4MPyP > TMAP ≫ T2MPyP). The limited reactivity of T2MPyP to RNA relative to that observed with the other porphyrin compounds may be attributed to the greater size of T2MPyP. One noteworthy exception of the reactivity profile of T2MPyP with RNA is shown in photomodification of stem IIb of the HIV-1 RRE RNA molecule. The modification of G7392 by T2MPyP observed at low ionic strength suggests that the internal loop that flanks G7392 may provide sufficient room to accommodate this porphyrin compound (Bartel et al., 1991; Mann et al., 1994; Battiste et al., 1994, 1995; Peterson et al., 1994).

The ionic strength dependence of the modification of DNA and RNA by porphyrin cations is quite different. High ionic strength abolished many sites of photomodification of $tRNA^{Phe}$ and the RRE RNA, whereas the corresponding photoreactions with three-way junction DNA complexes were found to be insensitive to ionic strength. The T4MPyP porphyrin molecule binds to double-stranded DNA by intercalation, and this interaction is disrupted by high ionic strength buffer conditions (Fiel, 1989; Nussbaum et al., 1994). The findings reported here and previous studies (Foster et al., 1988) suggest that classical modes of intercalation cannot explain the modification patterns observed for $tRNA^{Phe}$ and RRE RNA by the porphyrin cations. The major site of photomodification does not arise between base pair steps that would be consistent with a binding mode displayed by a typical intercalative agent (Dougherty & Pasternack, 1992). The specific folded conformation of the three-way junction DNA complex that enables porphyrins to bind with high affinity and specificity is apparently insensitive to KCl, whereas analogous porphyrin-binding pockets are modified or distorted in tRNA due to the stable formation of the RNA tertiary structure under these salt conditions. The HIV-1 RRE possesses little tertiary structure for RNA equilibrated under similar buffer conditions, as assessed by Fe(II)−EDTA cleavage studies.[5] We believe the most conservative interpretation of our porphyrin modification data for both RNA molecules is that RNA tertiary structure, *per se*, is not the basis for the observed protection of sites from photomodification. The formation of coaxially stacked RNA helices, an underlying determinant of RNA tertiary structure, is energetically favored in buffers containing molar concentrations of monovalent cations (Walter & Turner, 1994; Walter

[5] D. W. Celander and J. M. Nussbaum, unpublished observations.

et al., 1994). These ionic conditions lead to protection of the bases of helices by porphyrin photomodification; therefore, coaxial stacking of contiguous helices may directly interfere with porphyrin cation binding and subsequent photomodification of RNA. The noted similarities and differences in porphyrin reactivity toward RNA and three-way junction DNA may be rationalized once these porphyrin−nucleic acid complexes are studied by nuclear magnetic resonance spectroscopy and by crystallography.

Coaxial stacking is a contributing force in folding RNA molecules. The various modes whereby coaxial stacking is stabilized are currently becoming established. Structural motifs that directly stabilize coaxially stacked RNA helices may interfere with the utility of the described porphyrin ion photochemical method, thereby limiting its usefulness for studying folding transitions. One example of the method's limitation was revealed when we applied it to study the folded structure of the L-21 Sca I RNA derived from the self-splicing group I intron of *Tetrahymena thermophila*. The P4−P6 region of the intron is an independent folding domain (Murphy & Cech, 1993). The helical elements P4 and P6 are coaxially stacked within this domain, as assessed by electron microscopy (Murphy et al., 1994; Wang et al., 1994). The bases of the P4 and P6 helices are not modified by the T4MPyP porphyrin cation, even though other regions of the RNA molecule are extensively modified.[5] These data suggest that P4 and P6 may be coaxially stacked even in Tris-HCl/EDTA buffer; this interpretation is consistent with previous work with the P4−P6 domain in which these helices were found to be coaxially stacked in the absence of stabilizing counterions (Wang et al., 1994). The coaxial stacking of these helices alternately may be stabilized by additional base-triple interactions that pair nucleobases located in the J6/7 and J3/4 joining regions with base pairs in the P4 and P6 helices, respectively (Michel & Westhof, 1990; Michel et al., 1990; Green & Szostak, 1994; Chastain & Tinoco, 1992).

In conclusion, we have found that three cationic porphyrin molecules promote efficient photomodification of nucleobases in RNA domains that display the propensity to form helical stacking interactions. Junctions modified by these reagents may be tested further for their ability to adopt coaxially stacked helical domains by using additional experimental approaches such as helix fusion (Murphy et al., 1994) or electrophoretic mobility assay (Duckett et al., 1995). These porphyrin reagents also may be useful for monitoring the folding of coaxially stacked helices in other RNA molecules. The cationic porphyrin compounds described here should be amenable to structural studies of any RNA molecule for which a working RNA secondary structure model is available.

REGISTRY NUMBERS

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