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Preferential DNA Cleavage under Anaerobic Conditions by a DNA Binding Ruthenium Dimer

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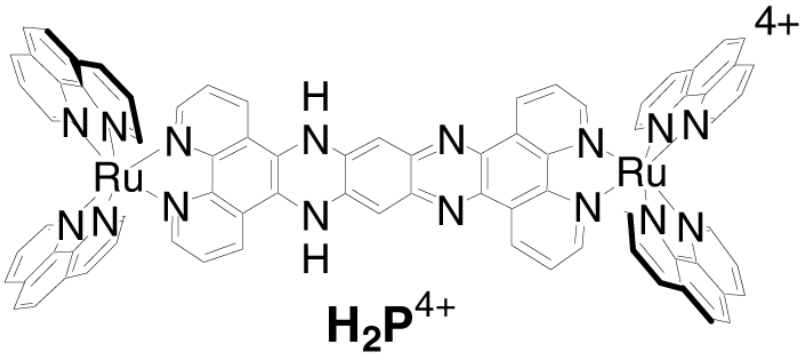
Abstract



In the absence of O_2, the cationic complex, $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$ ($\mathbf{P^{4+}}$), undergoes in situ reduction by glutathione (GSH) to form a species that induces DNA cleavage. Exposure to air strongly attenuates the cleavage activity, even in the presence of a large excess of reducing agent (e.g., 40 equiv GSH per $\mathbf{P^{4+}}$) suggesting the complex may be useful in targeting cells with a low oxygen microenvironment (hypoxia) for destruction via DNA cleavage. The active species is identified as the doubly reduced, doubly protonated complex $\mathbf{H_2P^{4+}}$ and a carbon-based radical species is implicated in the cleavage action.. We postulate that the pO_2 regulates the degree to which carbon radical forms and thus regulates the DNA cleavage activity.

The use of transitions metal complexes in medicine has enjoyed extensive attention given the tremendous success of cisplatin as a chemotherapeutic agent[1] and the ability of many metal complexes to interact with and damage cellular structures, particularly DNA.[2-7]

A large number of DNA cleaving metal complexes function via the activation of dioxygen to generate reactive oxygen species (ROS), such as hydroxyl radical and superoxide radical.[8,9] These ROS are ultimately responsible for the DNA cleavage. Others, including cisplatin and certain photoactivated,[10-14] oxidizing[15,16] or hydrolyzing complexes,[8] do not require O_2 to function, but they are also insensitive to the cellular $[O_2]$. Compounds that show enhanced cleavage activity under a low oxygen microenvironment (hypoxia) are rare[17-21] but offer a unique mechanism to target tumor cells under such conditions. These hypoxic tumor cells are often the most resistant to radiotherapy[22,23] and chemotherapy[24,25] and the most susceptible

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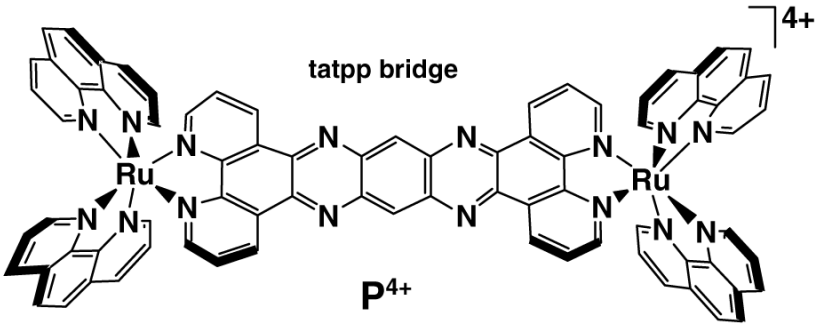
SUPPORTING INFORMATION AVAILABLE Procedures for the DNA cleavage assay and synthetic methods for preparation of [**P**][Cl]$_3$ and [$\mathbf{H_2P}$][Cl]$_4$ (one page). Absorption spectrum of GSH reduced $\mathbf{P^{4+}}$ (one page).

towards metastasis,[26,27] making this subpopulation a particularly attractive chemotherapeutic target.



We have discovered that the cationic ruthenium dimer, $[(phen)_2Ru(tatpp)Ru(phen)_2]^{4+}$ (**P**$^{4+}$) (tatpp = 9,11,20,22 -tetraazatetrapyrido[3,2-*a*: 2′,3′-*c*: 3″,2″-*1*: 2‴,3‴-*n*]-pentacene and phen = 1,10-phenanthroline) shown above (water soluble as the chloride salt) not only induces DNA cleavage in the presence of mild reducing agents but shows enhanced activity under anaerobic conditions. The fact that exposure to air attenuates the cleavage activity suggests that ROS are not responsible for the observed cleavage and that such a complex might be useful in targeting cells under hypoxic conditions. Complex **P**$^{4+}$ is known to intercalate and bind DNA tightly (K_b 1.1×10^7 M^{-1} at 25 mM NaCl).[28,29] The strong interaction with DNA is not unusual for this class of cationic complexes and it has a number of structural similarities to many known metallointercalators[13,14,30-33] including those that are know to thread their way through the DNA double-helix.[34]

The ability of **P**$^{4+}$ to cut DNA was examined by following the conversion of supercoiled plasmid DNA (form I) to the circular form (form II) or linear form (form III) using agarose gel electrophoresis to separate the products (experimental details given in ESI). As shown in Figure 1, **P**$^{4+}$ alone does not cause appreciable DNA cleavage (lane 2), however, addition of a mild reducing agent such as glutathione (GSH) leads to cleavage activity (lanes 4 &5). However the yield of cleavage products is clearly higher under anaerobic conditions (compare lane 4 vs lane 5) Yields of cleavage products (Forms II+III) under aerobic and anaerobic conditions are 55 % and 97 %, respectively.[35] The appearance of linear DNA in lane 5 appears to result from sequential ss cuts, not ds cleavage, thus the overall cleavage activity is single-strand (ss) sission.

Given the importance of excluding trace O_2 as playing a role in the observed cleavage activity, a positive control was included. Under anaerobic conditions, Iron(II)-Bleomycin (Fe-Blm) is known to induce DNA nicks but not ds cuts. When exposed to O_2, however, Fe-Blm is an effective ds-nuclease.[36-38] As seen in Figure 1, lane 6, Fe-Blm in the presence of O_2 causes extensive DNA-ds breaks whereas when O_2 was excluded (lane 7), only ss nicking is observed. These studies were carried out side by side with the **P**$^{4+}$ cleavage experiments demonstrating unequivocally that **P**$^{4+}$ is a more effective DNA cleaving agent under reducing and hypoxic conditions. Due to the known photoreactivity of **P**$^{4+}$, all experiments were conducted in the dark so that photochemically induced cleavage reactions could be ruled out. All cleavage experiments were conducted under low light conditions as **P**$^{4+}$ is known to be photoactive under some conditions.[39-41] Control experiments conducted in the dark or under ambient laboratory lighting gave identical results, showing that this cleavage reaction is not a photochemical reaction.

We have previously examined the redox chemistry of **P**$^{4+}$ in water at various pH's by electrochemical, spectroelectrochemical and chemical reduction methods.[39-41] Reaction 1 shows the two reduction products of **P**$^{4+}$ in water at pH 7.0. The redox reactions are reversible

and easily followed by visible spectroscopy as distinct changes in the absorption spectrum are observed for each reduction and protonation event. It is readily apparent that GSH reacts with $\mathbf{P^{4+}}$ as the solution color changes from yellow to green

$$\mathbf{P}^{4+} \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} \mathbf{P}^{3+} \underset{-e^- -2H^+}{\overset{+e^- +2H^+}{\rightleftharpoons}} \mathbf{H_2P}^{4+} \quad (1)$$

upon addition of the GSH. As seen in Figure 2, the absorption spectra of $\mathbf{P^{4+}}$ in aqueous buffer (pH 7.0) after addition of GSH is identical to that of $\mathbf{H_2P^{4+}}$ as prepared by stoichiometric reduction and protonation or electrochemically.[40,42] Thus, it appears that $\mathbf{P^{4+}}$ is a prodrug, which is converted to the $\mathbf{H_2P^{4+}}$ by in situ reduction.

In order to identify the chemical species responsible for the observed anaerobic cleavage and to rule out participation by glutathyl radical species, we examined the cleavage activity of the $\mathbf{P^{4+}}$, $\mathbf{P^{3+}}$ and $\mathbf{H_2P^{4+}}$(see ESI for synthetic procedures) under anaerobic conditions without GSH present.[40,42]

As seen in Figure 3, $\mathbf{P^{4+}}$ does not induce appreciable DNA cleavage under aerobic or anaerobic conditions (lanes 2 and 3). $\mathbf{P^{3+}}$ (lanes 4 and 5) does show some enhanced nicking ability, however, $\mathbf{H_2P^{4+}}$ is clearly the most potent nicking agent (lanes 6 and 7). As seen in lanes 6 and 7 (Figure 3), the amount of DNA cleavage increases with increased [$\mathbf{H_2P^{4+}}$] as would expected if this complex is the actual cleaving agent. Thus one simple explanation for the attenuated cleaving activity under aerobic conditions would be reoxidation of $\mathbf{H_2P^{4+}}$ to $\mathbf{P^{4+}}$. Exposure of an aqueous solution of $\mathbf{H_2P^{4+}}$ to air is known to result in a rapid reoxidation of this complex to $\mathbf{P^{4+}}$, as measured by UV-visible absorbtion spectroscopy.[39]

The mechanism of DNA cleavage is still unclear, however Yamaguchi and coworkers have shown that dihydropyrazines cleave DNA by both oxygen dependent and independent pathways.[43-46] $\mathbf{H_2P^{4+}}$ contains the dihydropyrazine substructure and thus could function in a similar manner with the high DNA binding affinity further enhancing its activity. For dihydropyrazines, the oxygen independent cleavage activity is attributed to the formation of a carbon-based radical on the drihydropyrazine moiety. Thus, we speculate that DNA bound $\mathbf{H_2P^{4+}}$ is cleaving DNA in a similar manner via the generation of reactive carbon-based radicals that are in close proximity to the DNA due to the tight DNA binding.

To test, this hypothesis, the cleavage activity of $\mathbf{H_2P^{4+}}$ was examined in the presence of various radical trapping and metal complexing reagents. DMSO is an effective scavenger of diffusible oxygen-based radicals such as ·OH, and superoxide.[47,48] On the other hand, TEMPO (2,2,6,6-tetramethyl-1-piperdinyloxy) is an effective scavenger of carbon radicals or metal-based radicals but ineffective with oxygen-based radicals.[49,50] As seen in Figure 3, addition of up to 5% DMSO by volume has no effect on the cleavage activity (lane 3) whereas addition of 2 mM TEMPO stops most of the DNA cleavage (lane 4). This data clearly supports the role of carbon-based radicals in the cleavage mechanism. Yamaguchi and coworkers postulated that trace metals ions, such as Cu^{2+}, activated the dihydropyrazines to the reactive form.[43-46] This does not seem to be the case here as added EDTA, at concentrations up to 1 mM, has little effect on the cleavage activity (Figure 4, lane 5) of $\mathbf{H_2P^{4+}}$. We are further investigating this unusual behavior and hope to elucidate the cleavage mechanism with the help of EPR spectroscopy. We note that the carbon-based radical species would likely be very reactive towards O_2 in solution and this 'quenching' reaction could also explain the observed sensitivity of this cleavage activity to oxygen.

It is interesting to observe that the loading of $\mathbf{P^{4+}}$ onto the DNA in the following studies (ranging from 1 complex per 12 DNA-bp to 5 complexes per DNA-bp) is relatively low compared to many metal-based DNA cleaving agents. In fact, the [Fe Blm] used in the cleavage experiment

shown in Figure 1 was purposefully kept the same as the [$\mathbf{P^{4+}}$] in the same experiment. As can be seen, the cleavage activity of $\mathbf{P^{4+}}$ under reducing, anaerobic conditions is comparable with Fe-Blm under aerobic conditions. To our knowledge, this is the first example of a metal complex with potentiated DNA cleavage activity under hypoxic conditions, suggesting potential therapeutic applications. Future studies will establish the mode of action and it effects on tumor cells in vitro.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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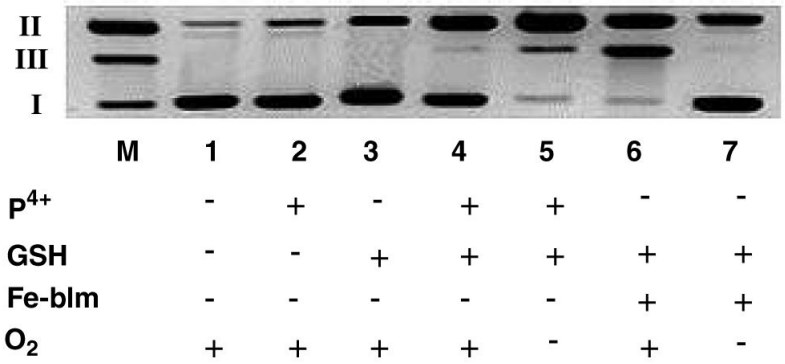

Figure 1.

Cleavage of supercoiled pUC18 DNA (0.154 mM) by $\mathbf{P^{4+}}$ in 7 mM Na_3PO_4 buffer (pH 7.0) at 25°C. Lane M, marker lane containing Form I, II and III DNA; lane 1, DNA control; lane 2, DNA + $\mathbf{P^{4+}}$ (0.0128 mM); lane 3, DNA + GSH (0.513 mM); lane 4, DNA + GSH (0.256 mM) + $\mathbf{P^{4+}}$ (0.0128 mM) under aerobic conditions; lane 5, same as lane 4 under anaerobic conditions; lane 6, DNA + GSH (0.512 mM) + Fe-Blm (0.0128 mM) under aerobic conditions; lane 7, same as lane 6 under anaerobic conditions. Incubation time for all these cases was limited for 1 hour.

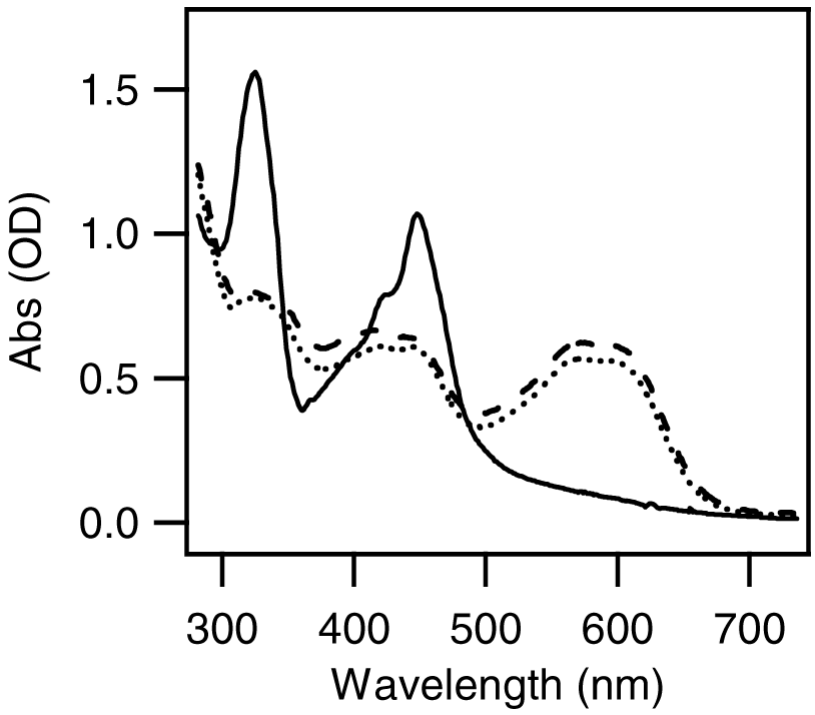

Figure 2.

Absorption spectra of $\mathbf{P}^{4+}$ (12.8 μM) before (solid line) and after (dotted line) addition of 10 equiv. GSH in anaerobic 7 mM Na_3PO_4 buffer (pH 7.0). Dashed line is the absorption spectrum of $\mathbf{H_2P}^{4+}$ in MeCN when prepared by stioichiometeric cobaltocene reduction and trifluoroacetic acid protonation.[42]

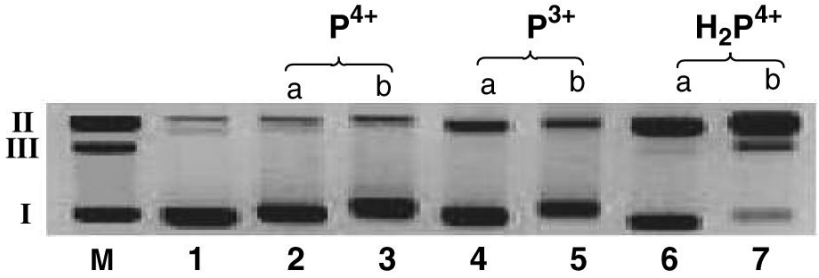

Figure 3.

Agarose gel of supercoiled pUC18 DNA (0.154 mM) in the presence of $\mathbf{P^{4+}}$, $\mathbf{P^{3+}}$ and $\mathbf{H_2P^{4+}}$. All incubations were performed under anaerobic conditions with an incubation time of 2 h at 25°C. The ratio of complex to DNA-bp was (a) 0.083 or (b) 0.20 as indicated above each lane. Lane M, marker; lane 1, DNA control; lane 2, DNA + $\mathbf{P^{4+}}$ (0.0128 mM); lane 3, DNA + $\mathbf{P^{4+}}$ (0.0307 mM), lane 4, DNA + $\mathbf{P^{3+}}$ (0.0128 mM); lane 5, DNA + $\mathbf{P^{3+}}$ (0.0307 mM), lane 6, DNA + $\mathbf{H_2P^{4+}}$ (0.0128 mM); lane 7, DNA + $\mathbf{H_2P^{4+}}$ (0.0307 mM).

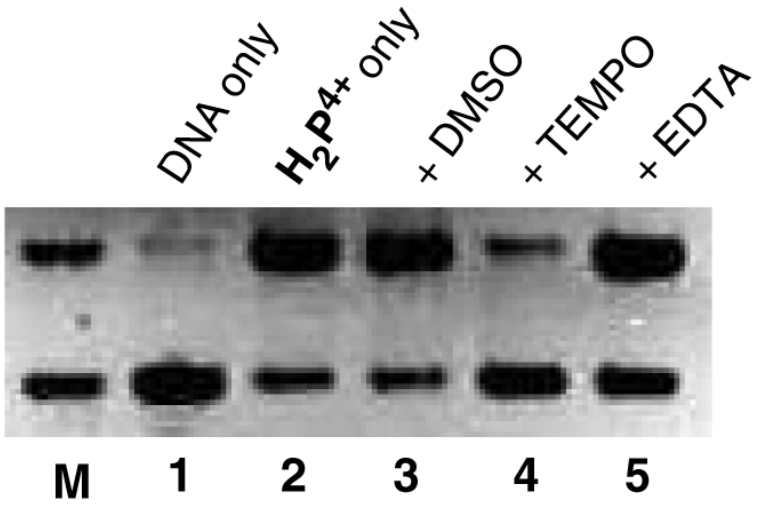

Figure 4.

Agarose gel of supercoiled pUC18 DNA (0.154 mM) in the presence of $\mathbf{H_2P^{4+}}$ (0.0256 mM). All incubations were performed in 7mM Na_3PO_4 buffer (pH 7.0) at 25^0C under anaerobic conditions with an incubation time of 2 h. The ratio of complex to DNA-bp was 0.16. Lane M, marker lane containing Form I and II DNA; lane 1, DNA control; lane 2, DNA + $\mathbf{H_2P^{4+}}$; lane 3, DNA + $\mathbf{H_2P^{4+}}$+ DMSO (0.64 M); lane 4, DNA + $\mathbf{H_2P^{4+}}$ + TEMPO (2.04 mM); lane 5, DNA + $\mathbf{H_2P^{4+}}$ + EDTA (1.02 mM).