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Reactivity of Bulged Bases in Duplex DNA with Redox-active Nickel and Cobalt Complexes

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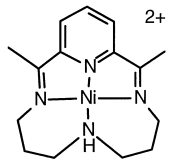
The square-planar, macrocyclic complex NiCR (CR = (2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]-heptadeca-1(17),2,11,13,15-pentaenato)) as well as $CoCl_2$ have been investigated as catalysts for the site-specific oxidation of bulged T, C, and A nucleotides in duplex DNA oligomers. Previous studies of NiCR using $KHSO_5$ as oxidant indicated that this combination of reagents successfully probes the accessibility of N7 of guanine residues in non-canonical DNA and folded RNA structures. In the present study, the order of reactivity of bulged bases in synthetic oligodeoxynucleotides with NiCR/$KHSO_5$ is shown to be G > C > T ~ A. Although the nickel complex generates a less-reactive oxidant than does cobalt, its selectivity for bulged bases is much greater, rendering it a useful probe of exposed Cs and Ts in addition to Gs in DNA and RNA structure.

Keywords: Nickel comples; Oxidation; DNA Bulges

INTRODUCTION

In the past decade, a series of macrocyclic nickel(II) complexes has been developed as probes of DNA and RNA structures in which exposed, extrahelical guanine residues are subject to metal-catalyzed oxidation leading to strand scission [1–3]. Among these, the square-planar complex NiCR (CR = (2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17),2,11,13,15-pentaenato)) represents one of the most reactive and convenient complexes for analysis of guanine stacking [4]. In a typical procedure, a micromolar strand concentration of [^{32}P] end-labeled DNA or RNA is allowed to react with a micromolar concentration of NiCR in the presence of ~ 100 μM $KHSO_5$ in phosphate or cacodylate buffer (pH 7), and the oxidized nucleic acid is subsequently treated with hot piperidine (0.2 M, 90°C, 30 min), or aniline in the case of RNA, to reveal sites of oxidation as strand breaks for analysis by denaturing polyacrylamide gel electrophoresis (PAGE) [5]. For single-stranded DNA or RNA, all guanines are nearly equally reactive, and Gs are approximately 10-fold more reactive than other bases. In duplex DNA, Gs were still the principal site of reaction although the overall reactivity was greatly diminished.



NiCR

The utility of NiCR is manifested in its high reactivity with solvent-exposed guanines, particularly when N7 of G is accessible for binding. For example, guanines present in base bulges or hairpin loops in either DNA or RNA sequences were highly subject to oxidation by NiCR/$KHSO_5$ while those in a Watson–Crick helix were largely protected from reaction [2]. NiCR successfully matched an NMR study [6] of a dynamic population of bulged G residues in a duplex in which one strand contained five successive Gs while the opposite strand contained four Cs [7]. Furthermore, the reactivity of NiCR with exposed Gs in $tRNA^{Phe}$ correlated well with the accessibility of N7 of those residues as analyzed by X-ray crystallography and computations [8–10]. The use of NiCR as a structural probe has extended to other laboratories who have investigated aberrant DNA and folded RNA structures [11–16].

For comparison, we have also studied the utility of $CoCl_2$-catalyzed oxidation of DNA and RNA again using monoperoxysulfate. Interestingly,

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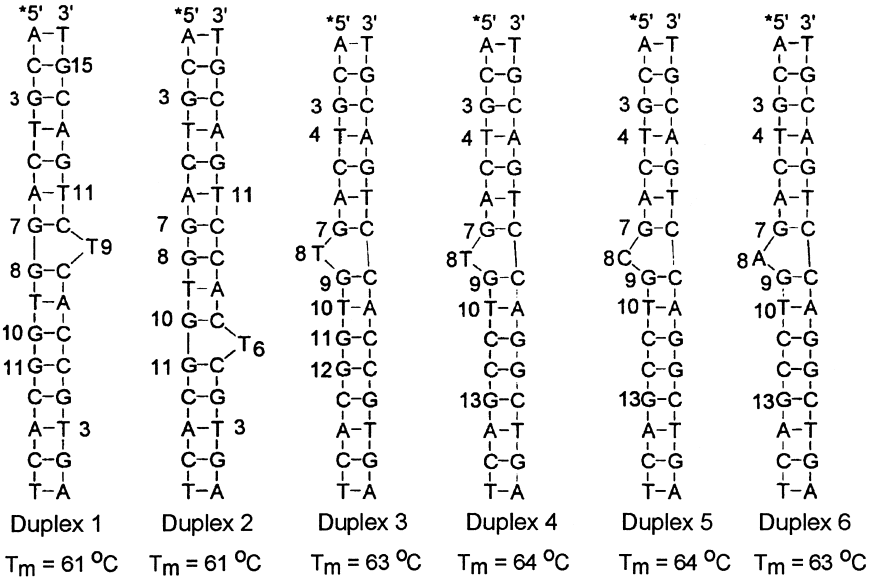

FIGURE 1 Sequences of bulged DNA duplexes used in this study. The strand indicated with an asterisk was 5′ end-labeled with ^{32}P and analyzed by PAGE. Melting temperatures for each duplex are indicated at the bottom.

$CoCl_2$- mediated reactions can also be used to probe guanine exposure in DNA and RNA [17] as well as the Z-DNA conformation [18] in which Gs are more highly exposed [19]; however, the reactivity of Gs with Co vs. Ni species is different. NiCR appears to react by generation of a metal-bound sulfate radical with different molecular recognition properties than that of the freely diffusible sulfate radical that is presumably formed from $CoCl_2$ [17,20].

Since guanine is the most electron rich of the nucleobases, it is both the most readily oxidized by a one-electron abstraction mechanism as well as the most readily attacked by electrophilic oxidants including most reactive oxygen species [21]. In the present work, we wished to ascertain whether or not nickel and cobalt-catalyzed oxidation of extrahelical residues could be extended to include other bases besides guanine. Toward this end, synthetic oligodeoxynucleotides were designed that placed T, C, and A as bulged bases in various sequence contexts, and the extent of oxidation at the site of the bulge compared to stacked G residues was analyzed as strand scission resulting from piperidine treatment [22]. Comparisons are also made to reactions in the absence of redox-active metals in which sulfate radical was produced by photolysis of dipersulfate.

EXPERIMENTAL SECTION

Materials and Instrumentation

Oligodeoxynucleotides were purchased from Oligos, Etc., Inc.; T4 polynucleotide kinase was purchased from New England Biolabs. $KHSO_5$ (Oxone) was purchased from Spectrum and $K_2S_2O_8$ from Aldrich. $CoCl_2{\cdot}6H_2O$ was purchased from Fisher Scientific and [γ-^{32}P]-ATP was from Amersham. NiCR as the diperchlorate salt was synthesized according to a literature procedure [23]. Scanning densitometry, T_m measurements, and optical density measurements of DNA were performed on a DU 650 spectrophotometer from Beckman Instruments. T_m measurements (260 nm) were conducted in cacodylate buffer using the same solution conditions given below for reactions.

General Procedure for Ni and Co-catalyzed Oxidation of DNA

The purified oligonucleotides were 5′ end-labeled with [γ-^{32}P]-ATP by T4 polynucleotide kinase using standard procedures [2]. For oxidation reactions, the labeled strand (3 μM, 1.8 nCi) was annealed to its complement (3 μM) for 3 min at 90°C in sodium phosphate or sodium cacodylate buffer (10 mM, pH 7) and allowed to cool to room temperature. Reaction mixtures (50 μl) contained 3 μM duplex, 3 μM NiCR or $CoCl_2$, 10 mM $MgCl_2$, 100 mM NaCl, and 10 mM NaH_2PO_4 or $Na(CH_3)_2AsO_2$ (pH 7). Reactions were initiated by the addition of 120 μM $KHSO_5$ (unless otherwise indicated) and conducted at room temperature for 30 min. Samples of the reaction mixture were quenched by addition of 2 μl of 250 mM EDTA and 2 μl of 50 mM HEPES followed by dialysis, piperidine treatment, and gel electrophoresis as previously described [4]. Band intensities on the gel were determined by scanning densitometry in which the area percentage given in the tables is the area of the desired peak divided by the total area of all peaks. Values given are the averages of at least three

TABLE I The relative reactivities of base residues in duplexes 1, 2, and 3 toward $KHSO_5$ oxidation catalyzed by Ni vs. Co

	NiCR (%)	$CoCl_2$ (%)
Duplex 1		
G3	not detected	0.34 ± 0.13
G7	1.57 ± 0.09	6.26 ± 1.33
G8	0.83 ± 0.22	3.20 ± 0.62
G10	1.22 ± 0.17	6.51 ± 0.71
G11	not detected	1.27 ± 0.31
Duplex 2		
G3	not detected	1.59 ± 0.69
G7	0.76 ± 0.44	5.85 ± 1.13
G8	0.23 ± 0.79	1.09 ± 0.16
G10	2.23 ± 0.22	12.22 ± 1.16
G11	1.14 ± 0.38	3.48 ± 0.78
Duplex 3		
G3	not detected	1.03 ± 0.11
G7	2.00 ± 0.83	9.48 ± 0.32
T8	3.13 ± 0.05	1.66 ± 0.24
G9	0.73 ± 0.08	3.51 ± 0.74
G11	2.22 ± 0.35	12.86 ± 0.69
G12	1.59 ± 0.85	3.29 ± 0.55

The data are presented as % area (the area associated with the desired peak divided by the total area associated with all peaks) as determined by scanning densitometry of cleavage bands on PAGE. "not detected" means that the peak is too weak to be detected by scanning densitometry.

separate experiments. The oxidation conditions were chosen to ensure "single-hit" conditions such that total reactivity was $< 30\%$.

Photochemical Oxidations Using $K_2S_2O_8$

Reaction conditions were identical to those described above with the omission of metal complexes and the replacement of $KHSO_5$ (Oxone) with $K_2S_2O_8$ (1 mM). Photolyses were conducted for 30 min using a 254-nm, 6-W UV lamp (UVP) at a distance of 12 cm. Sample work-up and analysis was identical to that described above.

RESULTS AND DISCUSSION

The oligodeoxynucleotide sequences shown in Fig. 1 were chosen by analogy to a closely related sequence already studied by us containing bulged G residues [2]. Duplexes **1**–**3** contain bulged Ts sandwiched between G–C pairs on either the C-containing strand (**1** and **2**) or the G-containing strand (**3**). T_m values were obtained for all six duplexes and were consistently in the range of 61–64°C, indicating that the duplexes are likely to be stable structures under the room-temperature conditions of the oxidation reactions. That the Ts were extrahelical in duplexes **1**–**3** was confirmed by a standard reaction with $KMnO_4$ which is known to show high selectivity for exposed thymine residues [24]. Duplexes **4**–**6** allow comparison of T vs. C vs. A in the same bulge site.

Recognition of Thymine Bulges vs. Stacked Guanines

Oxidation of DNA bases is dependent upon both the reactivity of the base as well as its accessibility to reagents. Although duplexes **1**–**3** do not contain any exposed guanine residues, it has been known for some time that multiple G sequences stacked in a B helix are highly susceptible to oxidation via a one-electron mechanism. Therefore, the bulged T residue was placed in two different sites on a C-rich strand, either between the G_7 and G_8 base pairs (duplex **1**), between the G_{10} and G_{11} base pairs (duplex **2**), or on the G-rich strand at a site analogous to duplex **1** (duplex **3**) (see Fig. 1). Oxidation of duplexes **1**–**3** by $KHSO_5$ was conducted in the presence of either 3 μM NiCR or 3 μM $CoCl_2$, and the results are shown in Table I. In terms of overall reactivity, $CoCl_2$-mediated reactions always showed substantially more total oxidation of the radiolabeled G-rich strand (indicated by *). On the other hand, NiCR was much more selective for oxidation of the bulged site (T_8 of duplex **3**).

For both metal catalysts, guanine oxidation occurred predominantly at the 5′ G of a stacked GG sequence in accordance with the observations of Saito and others that hole formation in the duplex is favored at this site [25]. Little, if any, oxidation was observed at G_3 which is flanked by pyrimidines. Interruption of stacked G·C pairs with a bulged T between the Gs resulted in increased reactivity of the Gs (see G_7 and G_9 of duplex **3** compared to G_7 and G_8 of duplex **2**), presumably due to increased accessibility of the bases to oxidants. This is also noticeable in comparing the relative reactivities of G_{10} and G_{11}, compared to G_7 and G_8, which are greater in duplex **2** compared to **1**. Interestingly, the 5′-GG stacking effect was still present in all duplexes including duplex **3** even though a bulged base was present. For example, typical 5′-G:3′-G reactivity ratios are about 5:1 for a canonical B helix, [20] while the bulged T, either in the same strand or opposite, reduced the reactivity ratio to about 3:1 for both catalysts in most cases. Thus, the bulged thymidine residue must be predominantly extrahelical in a fashion that permits stacking of guanines on either side of it with each other. This concept is fully consistent with NMR studies indicating that a T bulge in a poly-dA tract exists in a predominantly "looped out" conformation [26].

Overall, it appears that NiCR/$KHSO_5$ is an effective system for identifying bulged T residues even in the presence of relatively reactive stacked GG sequences. In contrast, $CoCl_2$ is too reactive with guanine bases stacked in the helix to be useful as a probe of thymine base exposure. The difference in these two complexes can be ascribed to differences in the nature of the reactive intermediate that carries

TABLE II The relative reactivities of base residues in duplexes 4, 5, and 6 toward $KHSO_5$ oxidation catalyzed by Ni or Co compared to $SO_4^{-\cdot}$

	NiCR (%)	$CoCl_2$ (%)	$S_2O_8^{2-}/h\nu$ (%)
Duplex 4			
G3	not detected	1.50 ± 0.73	not detected
G7	0.24 ± 0.19	6.71 ± 0.35	5.07 ± 1.37
T8	2.41 ± 0.01	2.58 ± 0.48	2.60 ± 0.58
G9	0.76 ± 0.14	3.29 ± 0.62	3.30 ± 1.38
G13	0.77 ± 0.07	8.17 ± 1.84	5.92 ± 2.86
Duplex 5			
G3	not detected	not detected	not detected
G7	0.87 ± 0.15	11.0 ± 0.70	5.19 ± 2.41
C8	4.89 ± 0.66	3.17 ± 0.34	2.15 ± 0.38
G9	0.46 ± 0.33	1.99 ± 0.34	2.06 ± 0.73
G13	0.49 ± 0.02	5.59 ± 0.72	4.96 ± 1.68
Duplex 6			
G3	not detected	not detected	not detected
G7	0.55 ± 0.21	13.74 ± 1.11	0.92 ± 0.39
A8	0.42 ± 0.15	0.70 ± 0.13	0.21 ± 0.04
G9	0.98 ± 0.06	0.98 ± 0.14	0.98 ± 071
G13	0.41 ± 0.18	5.60 ± 0.38	2.43 ± 0.80

The data are presented as % area (the area associated with the desired peak divided by the total area associated with all peaks) as determined by scanning densitometry of cleavage bands observed by PAGE. "not detected" means that the peak was too weak to be detected by scanning densitometry.

out oxidation. In the previous work, we showed that $CoCl_2$-catalyzed oxidation of guanine in DNA and RNA with $KHSO_5$ was consistent with the formation of freely diffusible sulfate radical, $SO_4^{-\cdot}$, that is quenched in the presence of added alcohols such as ethanol [17,27]. Similarly, we found that addition of 25 or 100 mM ethanol to $CoCl_2$-mediated oxidations of the duplexes studied here resulted in 50 and 70% decreases, respectively, in overall reactivity. On the other hand, ethanol had no effect on the reactivity of NiCR/$KHSO_5$ under the same conditions. As before, [17,27] we conclude that the species responsible for base oxidation in the nickel-catalyzed system involves a metal-bound oxidant, likely a high-valent nickel(III) species with a bound sulfate radical rather than a freely diffusible one. Such a species would be expected to be less reactive and more selective than free $SO_4^{-\cdot}$. In addition, binding to DNA bases may play a role in delivery of metal-bound oxidants to specific sites in DNA [7,10].

Comparison of T, C, and A Bulges

Duplexes **4**–**6** allow comparison of T vs. C vs. A in the same sequence context. The sequence selected was analogous to duplex **3** with the exception that the highly reactive $G_{11}G_{12}$ sequence was inverted to the opposite strand. Oxidation of these duplexes mediated by NiCR and $CoCl_2$ in the presence of $KHSO_5$ is presented in Table II. For comparison, $SO_4^{-\cdot}$ was also generated by photolysis of dipersulfate under conditions that gave a similar extent of overall reaction. As with duplexes **1**–**3**, the overall reactivity of duplexes **4**–**6** with $CoCl_2/KHSO_5$ was higher and the selectivity for the bulge site lower than with NiCR/$KHSO_5$. Examination of the reactivity of NiCR/$KHSO_5$ with duplex **5** again shows that this combination of reagents is able to effectively recognize C bulges since the reactivity of the C bulge compared to stacked Gs is typically >5:1. This compares favorably with another oxidation reagents, KBr + $KHSO_5$, previously developed in this laboratory for the selective recognition of C bulges in duplex DNA [28]. On the other hand, an A bulge in duplex **6** was not at all recognized by any of the oxidation systems.

Curiously, the data for sulfate radical-mediated oxidation of duplexes **4**–**6** only partially mirrors that of the $CoCl_2/KHSO_5$ system. Notable is the high reactivity of G_7 toward cobalt-mediated oxidation, which is not seen with either NiCR or photolysis of $S_2O_8^{2-}$. This may be an indication that Co^{II} binds to certain structural motifs in DNA thereby skewing the reactivity to selected sites. In any case, the high reactivity of nominally intrahelical guanines with $CoCl_2/KHSO_5$ is an indication that its use as a structural probe for bulge sites is limited to extrahelical Gs. On the other hand, NiCR/$KHSO_5$ successfully identified bulged T and C bases. The lack of reactivity of A could be due to any of the three factors: (i) an intrinsic lack of reactivity of the adenine base toward these oxidants, (ii) oxidation of A to non-piperidine labile products such that no strand scission is detected by gel electrophoresis, or (iii) intrahelical stacking of the adenine base between adjacent purines. In order to test these hypotheses, a single-stranded oligonucleotide that did not contain guanosine was studied.

Intramolecular Comparison of T, C, and A Reactivity

A 18-mer oligodeoxynucleotide containing only thymidine, cytosine, and adenosine with the sequence 5′-d(ATCTCACATCTACACTAT)-3′ was 5′-end labeled with [^{32}P] and subjected to oxidation with 3 μM NiCR and 60 μM $KHSO_5$ using standard buffer conditions. The densitometry scan obtained from gel electrophoretic analysis of sites of piperidine-induced cleavage is shown in Fig. 2. The order of reactivity was consistent over the temperature range 0–35°C and was C > T ~A in ratios of 4.3:1.5:1, respectively. Each base was approximately twice as reactive at 35°C compared to 0°C, but the relative reactivities remained the same.

That significant oxidation and cleavage was seen at A sites indicates that low reactivity of A toward NiCR/$KHSO_5$ cannot be the only explanation for the absence of reaction at the A bulge in duplex **6**.

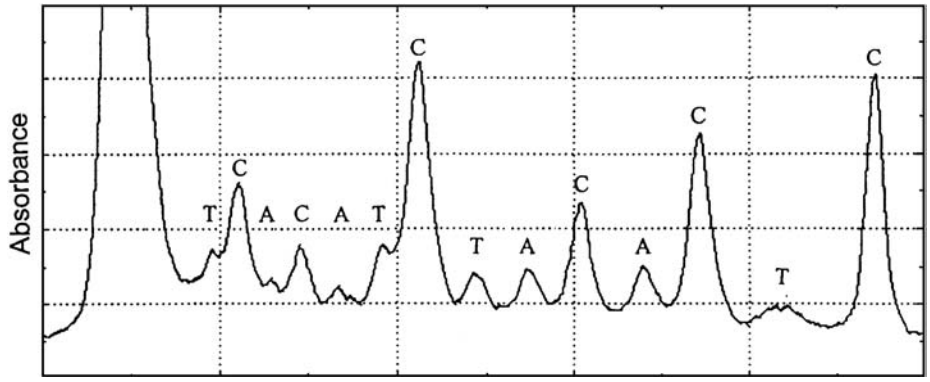

FIGURE 2 Densitometry scan of piperidine-induced cleavage after NiCR/$KHSO_5$ oxidation (at 35°C) of a non-guanine-containing oligodeoxynucleotide: 5′-d(ATCTCACATCTACACTAT).

Similarly, since most oxidized bases are piperidine-labile sites, [21] it is unlikely that A bulges are extensively oxidized without revealing cleavage. A more likely explanation for the low reactivity of the A bulge in **6** is low accessibility due to intrahelical stacking. An NMR study of an A bulge flanked by Gs showed intrahelical stacking of the adenine base at 25°C for both the single-base bulge as well as for an extended AAA bulge in the same sequence context, [29] consistent with our findings. Neither $CoCl_2$/$KHSO_5$ nor photolysis of dipersulfate revealed the bulged A site in **6**. Since both of the sulfate-radical producing oxidation systems are more reactive with DNA than is NiCR/$KHSO_5$, one might have expected higher reactivity at A_8 in duplex **6**. However, if $SO_4^{-\cdot}$ reacts primarily as an one-electron oxidant, the electron–hole generated could readily equilibrate to a G residue if the helix is well stacked.

Temperature Dependence of NiCR-catalyzed Oxidation of T, C, and A Bulges

While A is thought to preferentially stack into the helix when present at a bulge site, particularly in a purine tract, the nature of T and C bulges is less clear. For the smaller pyrimidines compared to purines, there is less driving force for intrahelical stacking of the hydrophobic surface of the base. Temperature-dependent NMR studies indicate that in the case of a C bulge located in a GCG sequence, both intrahelical and extrahelical forms exist in equilibrium with a preference for the extrahelical looped out structure at low temperatures (~0°C) compared to the stacked, intrahelical conformation at elevated temperatures (~40°C) [30]. To better characterize the duplexes presently under investigation, studies were conducted with NiCR/$KHSO_5$ over the temperature range of 0–35°C for duplexes **4**–**6**, and the results are shown in Table III.

The data in Table III suggest that the T bulge in duplex **4** and the C bulge in duplex **5** undergo temperature-dependent conformational changes preferring the looped-out form at 0°C in which the bulged base is more accessible to oxidant and becoming more intrahelical as the temperature is raised. The data must reflect conformational changes, because the reactivity pattern of NiCR/$KHSO_5$ has the opposite trend; reactivity with all bases increases by about a factor of two between 0 and 35°C. In contrast to T and C, the A bulge in duplex **6** appears to be stacked intrahelically at all temperatures studies. A is also rather unreactive toward oxidation, including NiCR/$KHSO_5$, but the single-stranded study described in the previous section found that A was intrinsically nearly as reactive as T.

CONCLUSIONS

Bulged nucleotides have been proposed to be important in frameshift mutagenesis, [31,32] and drug and protein binding has been shown in some cases to occur specifically at bulged sites [33–35]. Thus, a thorough understanding of the structure of bulged bases aids in painting a molecular picture

TABLE III The relative reactivities of bulged base residues in duplexes 4, 5, and 6 toward NiCR/$KHSO_5$ oxidation at various temperatures

Temperature	T_8 in duplex 4 (%)	C_8 in duplex 5 (%)	A_8 in duplex 6 (%)
0°C	12.93 ± 1.20	17.14 ± 1.59	0.42 ± 0.15
20°C	7.24 ± 1.10	7.84 ± 0.53	0.38 ± 0.09
35°C	4.04 ± 0.16	5.03 ± 0.27	0.30 ± 0.06

The data are presented as % area (the area associated with the desired peak divided by the total area associated with all peaks) as determined by scanning densitometry of cleavage bands observed by PAGE.

underlying the mechanisms of these phenomena. Although X-ray crystallography and NMR spectroscopy provide higher resolution structural profiles, chemical probes can provide a more rapid and convenient entry into the analysis of such structures. NiCR and $CoCl_2$ in conjunction with the peracid oxidant $KHSO_5$ have previously shown utility for analysis of the accessibility of guanine residues in folded DNA and RNA including bulged G sites [9]. In the present work, NiCR/$KHSO_5$ is extended to the study of bulged C and T residues. In comparison, the $CoCl_2$/$KHSO_5$ system shows higher reactivity but its preferences for bulged pyrimidines is often lower than Gs stacked in the duplex. This is attributed to the nature of the oxidant, proposed to be a freely diffusible sulfate radical in the case of $CoCl_2$. For the NiCR/$KHSO_5$ oxidation system, it is thought that a less reactive but more selective nickel-bound oxidant is formed. Bulged As are highly unreactive in this system, in part due to intrinsically lower reactivity, but also because of their tendency to be intrahelically stacked. This study underscores the idea that appropriately designed macrocyclic ligands for nickel(II) can tune the redox activity of the complex for selective molecular recognition of bulged nucleotides in DNA and RNA.

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