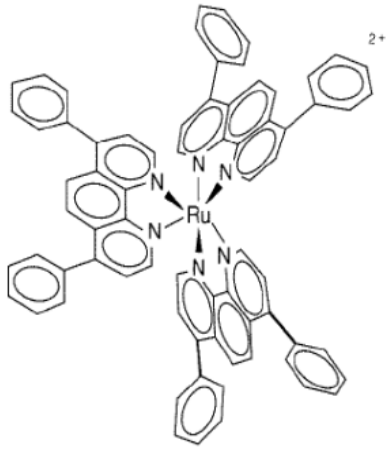
Spectroscopic Probes:

As discussed above, the tris(phenanthroline)ruthenium(II) complexes offer a novel spectroscopic probe of nucleic acids, since their luminescence is increased upon intercalation into the double helix. As a result the complexes provide a simple luminescent stain for DNA in fluorescent microscopy experiments. More interesting, perhaps, is the conformational selectivity of derivatives of tris(phenanthroline)ruthenium. $Ru(DIP)_3^{2+}$ (DIP =4,7-diphenyl-1,10-phenanthroline) shows enantiospecificity in binding to B-form DNA. Because of the steric bulk of the phenyl rings, detectable binding is seen only with the Δ-isomer in a righthanded helix; no binding is evident with the A-isomer. But with the left-handed Z-form helix, both isomers bind avidly. The shallow left-handed major groove can accomodate the two enantiomers, A left-handed but more B-like helix shows selectivity instead for the A-isomer. Spectroscopic experiments that measure the chiral selectivity of $Ru(DIP)_3^{2+}$ isomers in binding to a given DNA then provide a novel probe for helical handedness. Indeed, A-$Ru(DIP)_3^{2+}$ was the first spectroscopic probe for Z-DNA (or other alternate conformations that are sufficiently unwound to permit binding by the bulky left-handed isomer). Another set of derivatives of the tris(phenanthroline) metal complexes that may become exceedingly useful as spectroscopic probes are $Ru(bpy)_2dppz^{2+}$ and $Ru(phen)_2dppz^{2+}$ (dppz = dipyridophenazine). In these complexes the metal-to-ligand charge transfer is preferentially to the electron-accepting dppz ligand. In nonaqueous solutions, the complexes luminesce. However, in aqueous solution at pH 7, no luminescence is observed, likely because hydrogen bonding by water to the phenazine nitrogen atoms quenches the charge-transfer excited state. But the dppz ligand is also an expansive, aromatic heterocyclic ligand, and as a result both $Ru(bpy)_2dppz^{2+}$ and $Ru(phen)_2dppz^{2+}$ bind avidly to DNA by intercalation. Once intercalated, the phenazine ligand is protected from water.

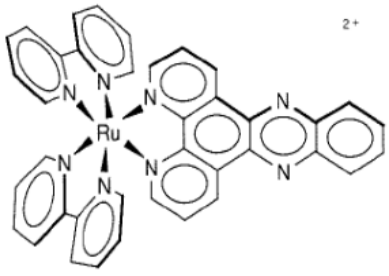
Therefore these complexes are luminescent when intercalated into DNA, whereas no luminescence is apparent from the complexes in the absence of DNA in aqueous solution. The enhancement factor is > 104 with DNA. One might consider the ruthenium complexes as true "molecular light switches" for DNA. Both simpler bipyridyl and phenanthroline derivatives as well as dppz complexes of ruthenium are currently being tethered onto other DNA binding moieties, in particular onto oligonucleotides, so as to develop new, nonradioactive luminescent probes for DNA sequences. These transition-metal complexes may provide the basis for the

development of new families of DNA diagnostic agents, and many industrial laboratories are currently exploring routes to accomplish these goals. Figure 11 illustrates A-$Ru(DIP)_3^{2+}$ and $Ru(bpy)_2$ dppz $^{2+}$, two complexes whose luminescence properties can be employed to probe nucleic acids.

Other transition-metal complexes besides those of ruthenium have shown some promise in spectroscopic applications with nucleic acids. Lanthanide ions have been applied both in NMR experiments and in luminescence experiments to probe tRNAs, and more recently with synthetic DNAs of differing sequence and structure. Lanthanide ions have been exceedingly useful in probing Ca^{2+} binding sites in proteins, and one would hope that a parallel utility would be achieved with nucleic acids. Their poor absorptivity have made luminescent experiments difficult, however, requiring relatively high concentrations of material. Nonetheless, the sensitivity of luminescent lifetimes to coordination and indeed solvation is providing a novel spectroscopic handle to explore binding sites and structures of the macromolecules. Another quite novel luminescent handle has been phenanthroline and diphenylphenanthroline complexes of copper(I). These complexes are extremely valuable cleavage probes, as we will see later; to characterize better their interactions with the helix, luminescence experiments are being explored. A problem here has been the nonphysiological conditions necessary to achieve detectable luminescence. Nonetheless, studies with the copper complexes demonstrate how the whole range of transition-metal chemistry and spectroscopy is beginning to be applied in sorting through nucleic- acid interactions.



Λ-$Ru(DIP)_3^{2+}$



$Ru(bpy)_2dppz^{2+}$

Two spectroscopic probes of nucleic acids: Λ-$Ru(DIP)_3^{2+}$ and $Ru(bpy)_2dppz$

Figure11

b.Metallo footprinting Reagents

Probably the most widespread application of metal nucleic-acid chemistry in the biology community has been the utilization of metal complexes for chemical footprinting. The footprinting technique (Figure 10) was developed by biologists as a means of locating protein-binding sites on DNA.^{32}P-end-labeled double-stranded DNA fragments could be digested with a nuclease, such as DNAse, in the presence or absence of DNA-binding protein. After electrophoresis of the denatured digests and autoradiography, one would find a "footprint," that is, the inhibition of cleavage by DNAse, at the spot bound by protein, in comparison to a randomly cleaved pattern found on the DNA in the absence of binding protein. Although DNAse is still widely used, this footprinting reagent has some disadvantages: (i) the nuclease is not sequence-neutral in its cleavage, resulting in lots of noise in the footprinting background; and (ii) since the nuclease is itself a large protein, its ability to provide high-resolution footprinting patterns of smaller molecules is quite limited. Several metal complexes now serve as high-resolution, sequence-neutral chemical footprinting reagents. Some of these reagents are shown in Figure 12. The first, as mentioned previously, was MPE-Fe(II)Y The complex con

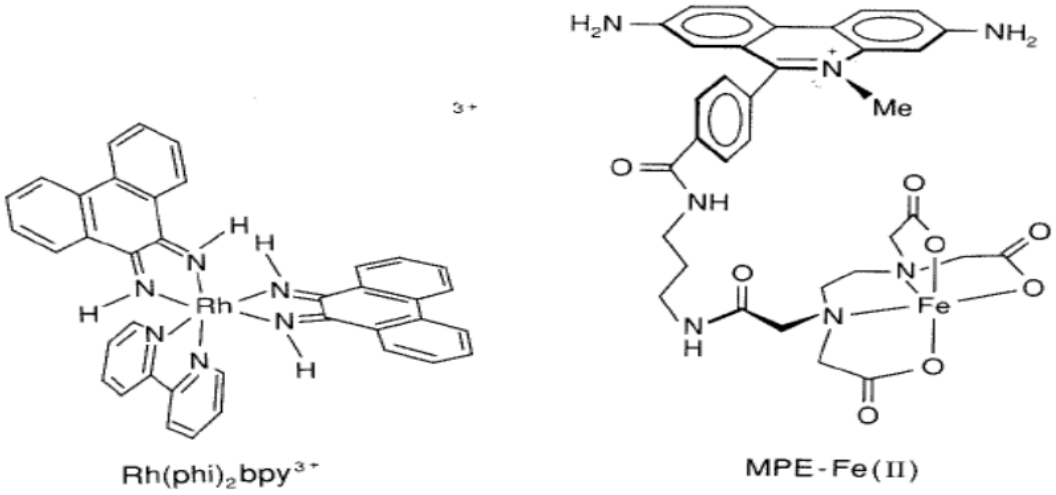


Figure 12

Examples of metallofootprinting reagents. $Rh(phi)_2bpy^{3+}$, a photofootprinting intercalator, and MPE-Fe(II), a sequence-neutral intercalating agent.

tains a sequence-neutral DNA binding moiety, the intercalator methidium, and a tethered DNA redox cleaving moiety, Fe(EDTA). The methidium, in binding nonspecifically to DNA, delivers the hydroxyl radicals, generated via Fenton chemistry at the Fe(II) center in the presence of peroxide and a reducing agent, to the DNA backbone in a random manner. Since the complex is small, high resolution can be achieved. Indeed, MPE-Fe(II) has been shown to footprint small natural products that bind to DNA, in addition to footprinting much larger.

DNA-binding peptides and proteins.

Perhaps simpler still and now very widely used as a footprinting reagent is $Fe(EDTA)^{2-}$ itself. The concept here is that $Fe(EDTA)^{2-}$, as a dianion, is unlikely to associate at all with the DNA polyanion. Hence hydroxyl radicals, generated via Fenton chemistry at a distance from the helix, would likely diffuse to the helix with a uniform concentration along the helix and provide a completely sequence-neutral pattern of cleavage. Tullius and coworkers have demonstrated this to be the case. The resolution is furthermore extremely high since the hydroxyl radical is sufficiently small that it can even diffuse within the DNA-binding protein to delineate binding domains. Some difficulties are found, however, with the high concentrations of activating reagents needed to activate a cleavage reagent that *does not* bind to the helix, and problems of course arise in trying to footprint metalloproteins. Nonetheless, $Fe(EDTA)^{2-}$, a reagent easily found on the biologist's shelf, is now finding great utility in labs as a chemical substitute for DNAse. Other transition-metal complexes are also finding applications in chemical footprinting. Both Cu(phen) + and manganese porphyrins have been used to footprint DNA-binding proteins. These complexes likely cleave DNA through either Fenton chemistry or direct reaction of a coordinated metal-oxo intermediate with the sugar-phosphate backbone. The complexes, however, appear to bind DNA predominantly along the surface of the DNA minor groove, and with some preference for AT-rich regions. The patterns obtained are actually quite similar to those found with DNAse, and thus the lack of high sequence neutrality is somewhat limiting. Furthermore, the complexes are most sensitive to binding moieties in the minor groove, rather

than those in the major groove, where proteins bind. Intercalators such as MPE-Fe(II) can sense binding species in both grooves. $Cu(phen)^{2+}$ has nonetheless proved to be quite effective in detecting hyperreactivities in the minor groove, owing to DNA structural perturbations that arise from protein binding in the major groove. Whether this sensitivity emanates from the intimate interaction of $Cu(phen)^{2+}$ in the minor groove of the helix, or because of the characteristics of the reactive radical formed, is not known.

Inorganic photochemistry has also been applied in developing metal complexes as photo footprinting reagents. Uranyl acetate, for example, at high concentrations, upon photolysis, promotes DNA cleavage. It is thought that the ions interact with the phosphates, generating some excited-state radical chemistry, although no detailed characterization of this chemistry has been undertaken. The cleavage reaction is nonetheless remarkably sequence-neutral and therefore shows some promise for photofootprinting applications. In fact, the applicability of uranyl acetate typifies how simple coordination chemistry and now even photochemistry may be helpful in the design of a variety of reagents that interact and cleave DNA, both nonspecifically and specifically. The biochemical techniques used to monitor such processes are sufficiently sensitive that even quite inefficient reactions in solution can be harnessed in developing useful reagents. The better our understanding of the chemistry of the coordination complex, the more effectively it may be utilized.

The best derivative of a tris(phenanthroline) metal complex currently being applied in footprinting experiments is $Rh(phi)_2bpy^{3+}$, a second-generation derivative of the tris(phenanthroline) series that binds DNA avidly by intercalation and in the presence of light promotes direct strand cleavage by hydrogenatom abstraction at the C3'-position on the sugar. Since no diffusing intermediate is involved in this photocleavage reaction, the resolution of the footprinting pattern is to a single nucleotide. Here the excited-state transition-metal chemistry involves a ligand-to-metal charge transfer, producing a phi cation radical that directly abstracts the hydrogen from the sugar at the intercalated site.

The high efficiency of this photoreaction and high sequence-neutral binding of the complex to double-stranded DNA add to the utility of this reagent in footprinting studies. Indeed, both DNA-binding proteins, bound in the major groove, and small natural products, associated with the minor groove, have been footprinted with $Rh(phi)_2bpy^{3+}$ to precisely that size expected

based upon crystallographic results. One may hope that this and other photofootprinting reagents will soon find applications for footprinting experiments *in vivo*.

Conformational Probes:

Metal complexes are also finding wide application in probing the local variations in conformation that arise along nucleic-acid polymers. X-ray crystallography has been critical in establishing the basic conformational families of doublehelical DNA, and to some extent how conformations might vary as a function of nucleic-acid sequence. Yet many conformations have still not been described to high resolution, and only a few oligonucleotides have been crystallized. Other techniques are therefore required to bridge the small set of oligonucleotide crystal structures that point to plausible structures and the large array of structures that arise as a function of sequence on long helical polymers. Furthermore, only a very small number of RNA polymers has been characterized crystallographically; hence other chemical methods have been needed to describe the folding patterns in these important biopolymers. Metal complexes, mainly through specific noncovalent interactions, appear to be uniquely useful in probing the structural variations in nucleic acids.

Nonspecific reactions of transition-metal complexes:

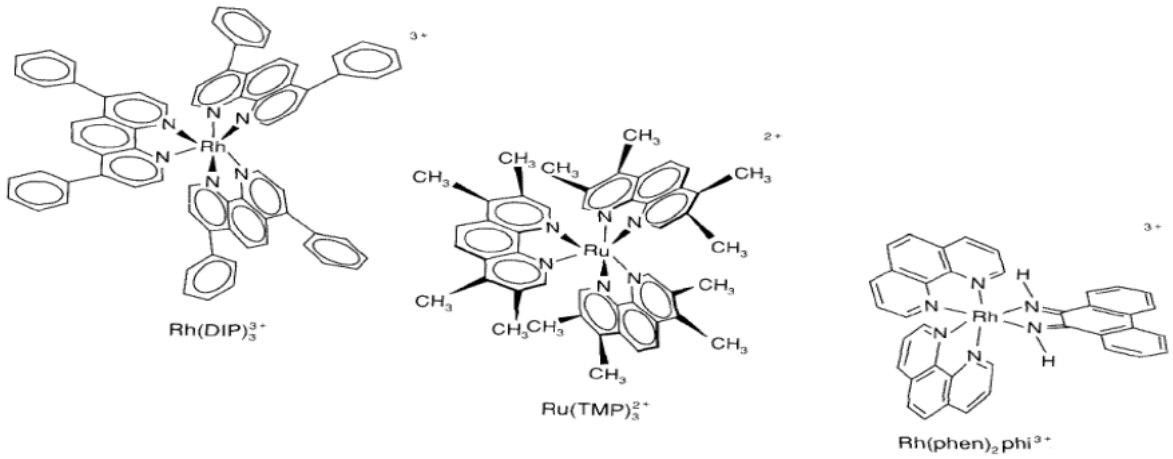
Hydroxyl radical cleavage with $Fe(EDTA)^{2-}$ illustrates again how simple metal complexes can be used in characterizing nucleic acids. One example involves efforts to describe the local structural variations in "bent" DNA. Biochemists had found that DNA fragments containing runs of adenines, such as in the tract dAAAAAA, possessed unusual gel-electrophoretic mobilities. Indeed, kinetoplast DNA isolated from mitochondria of trypanosomes showed a remarkable lacework pattern of structure, with loops and circles of DNA; these structures were found to be governed by the placement of these d(A) tracts. By constructing a series of oligonucleotides with adenine runs positioned either in or out of phase relative to one another, researchers found that the adenine tracts caused a local bending of the DNA toward the minor groove. But what were the detailed characteristics of these bent sites? Using hydroxyl radical cleavage of DNA, generated with $Fe(EDTA)^{2-}$, Tullius and coworkers found a distinctive pattern of cleavage across the adenine tracts, consistent with a locally perturbed structure. Here the notion again was that $Fe(EDTA)^{2-}$ in the presence of peroxide would generate hydroxyl radicals at a distance from the helix, and thus careful densitometric analysis of the cleavage across ^{32}P-end-Iabeled DNA fragments would reveal any differential accessibility of sugar residues to cleavage mediated by

the radicals caused by the bending. The cleavage patterns suggested a smooth bending of the DNA across the tract and indicated furthermore an asymmetry in structure from the 5'- to 3'-end of the adenine run.

The reactivities of other transition-metal reagents have also been used advantageously in probing nucleic-acid structures. As described $Os0_4$ reacts across the 5,6 position of accessible pyrimidines to form a cis-osmate ester. Upon treatment with piperidine, this base modification can yield scission of the sugar-phosphate backbone. Hence DNAs containing unusual local conformations with prominent solvent-accessible pyrimidines can be probed with $Os0_4$. The junction regions of Z-DNA, the single-stranded loops in cruciform structures, and a segment of the dangling third strand in H-DNA, have all been probed by means of the differential reactivity of osmium tetroxide with DNA sites dependent upon their accessibility. Surely other transition-metal chemistry will become similarly applicable.

Transition-metal complexes as shape-selective probes

Transition-metal complexes have also been designed with three-dimensional structures that target complementary structures along the helical polymer. This recognition of DNA sites, based upon *shape selection,* has proved to be extremely useful both in demarcating and in characterizing structural variations along the polymer and in developing an understanding of



Shape-selective probes that target local DNA conformations. $Rh(DIP)_3^{3+}$, which with photo-activation promotes double-stranded cleavage at cruciform sites; $Ru(TMP)_3^{2+}$, a photoactivated probe for A-like conformations; and $Rh(phen)_2phi^{3+}$, which targets openings in the DNA major groove.

Figure13

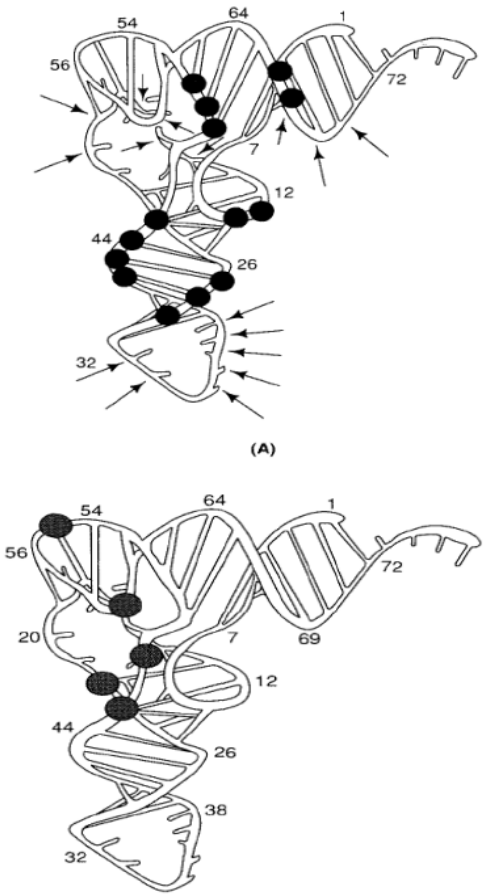
those factors important to the recognition of specific polynucleotide sites. Complexes, basically derivatives of the tris(phenanthroline) metal series, have been designed that specifically target A- and Z-form helices, cruciforms, and even subtle variations such as differential propeller twisting within B-form DNA. lie By appropriate substitution of the metal at the center of the coordinatively saturated complex, complexes that cleave the DNA at the binding site are obtained. Figure 13 shows some of these shape-selective conformational probes.

One example of this shape-selective cleavage is apparent in reactions of $Ru(TMP)^{2+}$ (TMP = 3,4,7 ,8-tetramethylphenanthroline) , a probe of the A-conformation. The complex was designed by incorporating methyl groups about the periphery of each phenanthroline ligand to preclude intercalative binding of the complex to the helix, owing to the bulkiness of the methyl groups, and at the same time to promote hydrophobic groove binding, Importantly, however, this hydrophobic groove binding could not occur against the minor groove of B-DNA, given the width and depth of the groove versus the size of the complex. Instead, the shape of the complex was matched well to the shallow minor-groove surface of an A-form helix. Binding studies with synthetic polynucleotides of A, B, and Z-form were consistent with this scheme. Photolysis of the ruthenium complex, furthermore, as with $Ru(phen)_3^{2+}$, leads to the sensitization of singlet oxygen, and hence, after treatment with piperidine, to strand cleavage. Thus, photocleavage reactions with $Ru(TMP)_3^{2+}$ could be used to delineate A-like regions, with more shallow minor grooves, along a helical polymer. At such sites, $Ru(TMP)_3^{2+}$ would bind preferentially, and upon photolysis, generate *locally* higher concentrations of singlet oxygen to mediate cleavage of the sugar-phosphate backbone. This scheme revealed that homopyrirnidine stretches along the helix ado pt a more A-like conformation.

The targeting of altered conformations such as Z-DNA has been described earlier in the context of a spectroscopic probe, $A\text{-}Ru(DIP)_3^{2+}$. Substitution of a photoredox-active metal into the core of the tris(diphenylphenanthroline) unit leads also to a complex that both binds and, with photoactivation, cleaves at the altered conformation. Both Co(III) polypyridyl and Rh(III) polypyridyl complexes have been shown to be potent photooxidants. Coupled to site-specific DNA binding, these metal complexes, with photoactivation, become conformationallys selective DNA cleavage agents. $Co(DIP)_3^{3+}$, for example, has been shown to cleave specifically at Z-form segments inserted into DNA plasmids. Perhaps even more interesting, on both natural plasmids and viral DNAs, the various sites cleaved by $Co(DIP)_3^{3+}$, corresponding both to Z-form sites and

to other locally altered non-B-conformations, coincide with functionally important regions of the genome, e.g., regulatory sites, gene termination sites, and intron-exon joints. The altered structures recognized by the metal complexes, therefore, appear to mark biologically important sites, those presumably recognized also by cellular proteins. Cleavage studies with these metal complexes, therefore, are providing some insight also into how Nature specifically targets and accesses the sequence information encoded along the DNA polymer, sequence information encoded indirectly through local structure.

The most striking example of the specificity to be derived from shape-selective targeting has been given by the double-stranded cleavage induced by $Rh(DIP)_3^{3+}$ at cruciforms. $Rh(DIP)_3^{3+}$, like its Co(III) and Ru(II) congeners, binds to locally unwound, non-B-conformations such as Z-DNA, but interestingly this potent photooxidant yields the specific cleavage of *both* DNA strands vs As mentioned above, describing the three-dimensional structures of RNAs is an even more complicated task than it is for double-stranded DNAs. Only a few tRNAs have been characterized crystallographically to high resolution, and for other larger RNA structures, such as 5S RNA, or any of the catalytic intervening- sequence RNAs, little is known about their folding characteristics. To understand the regulation and catalytic functions of these biopolymers, we need to develop chemical tools to explore these structures. Figure14 shows the results of cleavage studies using the variety of transition-metal probes on tRNA Phe. Hydroxyl-radical cleavage mediated by $Fe(EDTA)^{2-}$ reveals the protection of solvent-inaccessible regions, the "inside" of the molecule. MPE-Fe(II) appears to demarcate the double-helical regions, $Cu(phen)_2^{+}$ shows the loopedout single-stranded segments, and $Rh(phen)_2phi^{3+}$ seems to delineate those regions involved in triple-base interactions, the sites of tertiary folding. Taken together, the full structure of the tRNA can be described based upon cleavage data with transition-metal complexes. It therefore seems as if this full family of coordination complexes might be generally useful in delineating RNA structures. Still more work is needed quantitatively to compare the patterns obtained with the few well-characterized structures. Nonetheless, an important role for these and possibly other transition-metal reagents is indicated.



The diversity of cleavage sites for metal complexes on $tRNA^{Phe}$. In (A) is shown cleavage by probes that primarily detect features of RNA secondary structure. $Cu(phen)_2^+$ (arrows), detecting single-stranded regions and MPE-Fe(II) (black dots), detecting double-helical segments. In (B) are shown probes that detect protected or more complex structures on tRNA. Inaccessible sites protected from $OH\cdot$ after treatment with $Fe(EDTA)^{2-}$ are shown as shaded portions of the molecule, and specific cleavage by $Rh(phen)_2(phi)^{3+}$ at tertiary folds is indicated by the circles.[11b]

Figure14

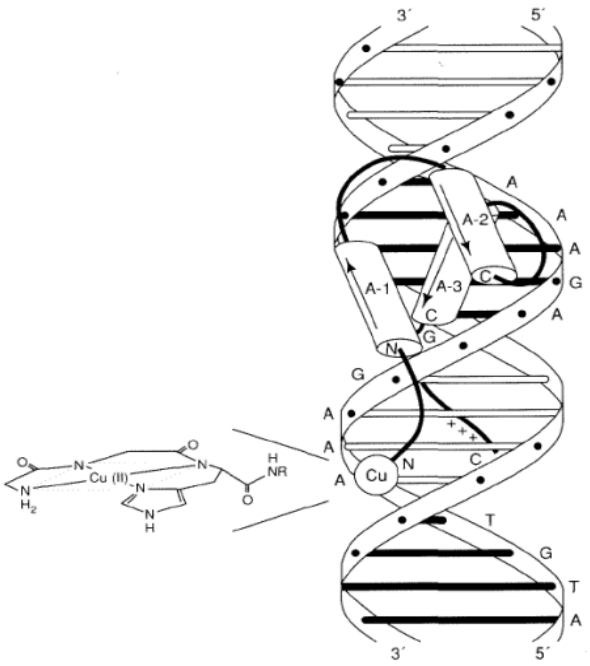
Other Novel Techniques

Transition-metal ions can also be used advantageously tethered onto peptides, proteins, oligonucleotides, and other natural products, to provide a chemical probe for their binding interactions with nucleic acids. This strategy, termed *affinity cleavage,* was developed by Dervan and coworkers in preparing and characterizing distamycin-Fe(II)EDTA. Distamycin is a known natural product that binds in the minor groove of DNA at AT-rich sequences. By tethering Fe(II)EDTA onto distamycin, the researchers converted the DNA-binding moiety into a DNA-cleaving moiety, since, as with MPE-Fe(II), in the presence of peroxide and a reductant,

hydroxyl radical chemistry would be delivered to the distamycin binding site. Unlike MPE-Fe(II) , however, the distamycin moiety shows preferential binding at some sites along the polymer, and hence only at those sites would the local hydroxyl-radical concentration be increased and cleavage be obtained. As a result the tethered $Fe(EDTA)^{2-}$ could be used as a cleavage probe, marking sites of specific binding.

Affinity cleaving has been generalized so that now $Fe(EDTA)^{2-}$ can be tethered onto both oligonucleotides and peptides to follow their interactions with nucleic acids. The sequence-specific binding of oligonucleotides to double-helical DNA through triple-helix formation is but one of many examples where the tethering of $Fe(EDTA)^{2-}$ has been applied advantageously. Other redox-active metals can be incorporated into DNA-binding moieties as well. Schemes have been developped to functionalize accessible lysine residues on DNA-binding repressor proteins with phenanthrolines, so that in the presence of copper ion, peroxide, and a reductant, the phenanthroline-bound copper on the protein would induce DNA strand cleavage. Through this scheme, again the conversion of a DNA-binding moiety into a cleaving moiety by incorporation of a redox-active metal, the specific binding sites of repressor proteins can be readily identified (far more quickly on large DNA than through footprinting).

Another scheme, which perhaps takes advantage more directly of bioinorganic chemistry, involves engineering redox metal-binding sites into DNA-binding proteins and peptides. The DNA-binding domain of the protein Hin recombinase was synthesized chemically, and first, to examine the folding of the peptide on the DNA helix, EDTA was tethered onto the peptide for Fe(II) cleavage experiments. But as is illustrated repeatedly in these chapters, Nature has already provided amino-acid residues for the chelation of metal ions into proteins. Thus the DNA-binding domain of Hin recombinase was synthesized again, now including at its terminus the residues Gly-Gly-His, a known chelating moiety for copper(II). This chemically synthesized peptide, with now both DNA-binding and DNA-cleaving domains, as illustrated in Figure 15, specifically promotes cleavage at the Hin recombinase binding site in the presence of bound copper and ascorbate. Interestingly, the addition of nickel(II) also leads to specific strand cleavage, without diffusible intermediates. Using this approach, taking advantage of the chelating abilities of amino acids and the cleaving abilities of different metal ions, one may prepare new synthetic, functional metalloproteins that bind and react with DNA.



A schematic of a synthetic DNA-cleaving peptide bound to DNA that was constructed by synthesis of the DNA-binding domain of Hin Recombinase with Gly-Gly-His at the N-terminus to coordinate copper.[80] Reproduced with permission from Reference 80.

FIGURE15

NATURE'S USE OF METAL/NUCLEIC-ACID INTERACTIONS

In the context of what we understand about the fundamental interactions and reactions of metal ions and complexes with nucleic acids, and also in comparison to how chemists have been exploiting these interactions in probing nucleic acids, we can also consider how Nature has taken advantage of metal ions in the construction of metalloproteins, nucleic-acid assemblies, and smaller natural products containing metal ions that interact with DNA and RNA.

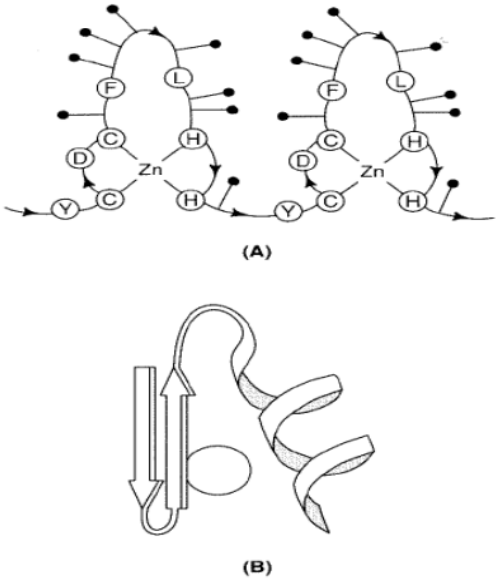
A Structural Role

One of the chief functions attributed to metal ions in biological systems is their ability to provide a structural center to direct the folding of a protein. Just as shape-selective recognition has been helpful in targeting metal complexes to specific sites on DNA, it appears that one element of the recognition of sites by DNA regulatory proteins may also involve the recognition of complementary shapes. Furthermore, metal ions appear to be used in these proteins to define the shape or folding pattern of the peptide domain that interacts specifically with the nucleic acid.

The DNA-binding metalloproteins that have received the greatest attention recently have been the "zinc-finger" regulatory proteins. It was discovered in 1983 that zinc ions played a role in the functioning of the nucleic acid-binding transcription factor IlIA (TFIIIA) from *Xenopus laevis,* which binds specifically both DNA, the internal control region of the 5S rRNA gene, and RNA, the 5S RNA itself. The protein (actually the 7S storage particle) was found to contain two to three equivalents of zinc ion. Dialysis removed both the associated zinc ions and the nucleic-acid-binding ability of the protein. Importantly, treatment with zinc ion, or in later studies with higher concentrations of Co^{2+}, restored the specific binding ability. Hence, zinc ion was shown to be functionally important in these eukaryotic regulatory proteins. The notion of a "zinc-finger structural domain" was first provided by Klug and coworkers, after examination of the amino-acid sequence in TFIIIA. It was found that TFIIIA contained nine imperfect repeats of a sequence of approximately 30 amino acids, and furthermore that each repeat contained two cysteine residues, two histidine residues, and three hydrophobic residues, in conserved positions. In addition, subsequent metal analyses were revealing higher zinc contents (7 to 11 equivalents) associated with the protein, and proteindigestion experiments indicated that several repeated structural domains existed in the protein. The two cysteine thiolates and two histidine imidazoles in each repeated domain could certainly serve to coordinate a zinc ion. Thus it was proposed that each peptide repeat formed an independent nucleic-acid-binding domain, stabilized in its folded structure through coordination of a zinc ion. The peptide unit was termed a "zinc finger," which is illustrated schematically in Figure 16. TFIIIA was therefore proposed to contain nine zinc fingers, which would cooperatively bind in the internal control region of the 5S RNA gene.

An enormous number of gene sequences from a variety of eukaryotic regulatory proteins was then found to encode strikingly similar amino-acid sequences, and many were dubbed zinc-finger proteins. The bioinorganic chemist, however, should be aware that chemical analyses supporting such assignments are first required. Nonetheless, several legitimate examples of eukaryotic nucleic- acid-binding zinc-finger proteins containing multiple zinc-binding peptide domains have emerged since the first study of TFIIIA, including the proteins Xfin from *Xenopus,* the Kruppel protein from *Drosophila,* the Spi transcription factor, and human testes-determining factor. It has therefore become clear that *the zinc-jinger domain represents a ubiquitous structural motif for eukaryotic DNA-binding proteins.*

What is the structure of a zinc finger, and how is this structure important for binding a specific nucleic-acid site? Based on a search of crystallographic databases for metalloproteins and an examination of the consensus sequence emerging for zinc fingers (that is, which residues were truly conserved and common to the different putative zinc fingers), Berg proposed a three-dimensional structure for a zinc finger, shown schematically in Figure 16. The proposed structure included the tetrahedral coordination of zinc by the two cysteine and histidine residues at the base of the finger and an a-helical region running almost the length of the domain. EXAFS studies also supported the tetrahedral zinc site. Since this proposal, two detailed two-dimensional NMR studies have been reported that are consistent with the tetrahedral zinc coordination and the a-helical segment. More recently, a crystal structure of a three-finger binding domain associated with an oligonucleotide was determined. The zinc fingers lie in the major groove of DNA, the a-helical region being within the groove. Not surprisingly, given basic coordination chemistry, the zinc does not interacts directly with the nucleic acid. Instead, the zinc ion must serve a structural role, defining the folding and three-dimensional structure of the protein scaffolding about it. This structure, defined by the metal at its center, like other coordination complexes, is able to recognize its complementary structure on the nucleicacid polymer.



(A) A schematic of a zinc-finger peptide domain.[82] (B) The proposed schematic structure of a zinc-finger domain based on comparisons to other structurally characterized metalloproteins.[85]

FIGURE 16

It should also be noted that this zinc-finger structural motif is not the only metal-containing or even zinc-containing structural motif important in nucleicacid- binding proteins.A clearly different domain is evident in the protein *GAL4,* a transcription factor required for galactose utilization in S. *cerevisiae.* A recent crystal structure of the protein bound to an oligonucleotide shows the protein to bind to DNA as a dimer; each monomer contains a binuclear zinc cluster with two zinc ions tetrahedrally coordinated by six cysteines (two cysteines are bridging), not dissimilar from proposed structures in metallothionein. Still another structural motif is found in the glucocorticoid receptor DNA-binding domain. Crystallography has revealed that this domain also binds DNA as a dimer; here each monomer contains two zinc-nucleated substructures of distinct conformation. The zinc ions are each tetrahedrally coordinated to four cysteine residues. Likely this too represents another structural motif for proteins that bind nucleic acids, and one again in which the metal serves a structural role.

Lastly, one might consider why zinc ion has been used by Nature in these nucleic-acid binding proteins. Certainly, the natural abundance of zinc is an important criterion. But also important is the absence of any redox activity associated with the metal ion, activity that could promote DNA damage [as with Fe(II) or Cu(II), for example]. In addition, other softer, heavier metal ions might bind preferentially to the DNA bases, promoting sequence-specific covalent interactions. Zinc ion, therefore, is clearly well-chosen for the structural center of these various nucleic-acid-binding proteins.

A Regulatory Role

Metalloregulatory proteins, like the transcription factors described above, affect the expression of genetic information through structural interactions that depend upon the metal ions, but unlike the zinc-finger proteins, metalloregulatory proteins act as triggers, repressing or activating transcription given the presence or absence of metal ion. In some respects, even more than zinc fingers, these systems resemble the Ca^{2+} activated proteins

Consider the biological system that must respond to changing intracellular metal concentrations. At high concentrations many metal ions become toxic to the cell; hence, a full system of proteins must be synthesized that will chelate and detoxify the bound-metal-ion pool. In order to actively engage these proteins, the genes that encode them must be rapidly transcribed. But at the same time, the DNA itself must be protected from the high concentrations of metal ion. Hence the need for these metalloregulatory proteins, which bind DNA in the

absence of metal ion, usually repressing transcription, but in the presence of metal ion bind the metal ion tightly and specifically, and as a consequence amplify transcription.

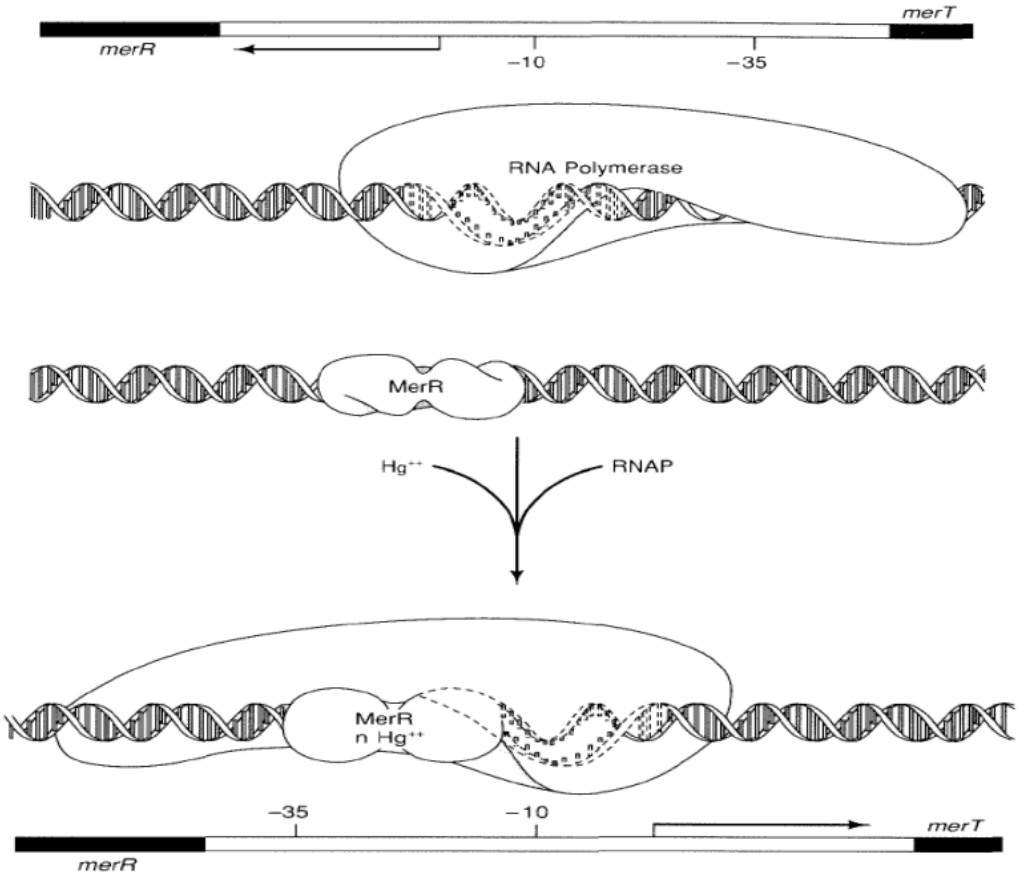
Perhaps the best-characterized metalloregulatory system thus far is the MerR system, regulating mercury resistance in bacteria. An inducible set of genes arranged in a single operon is under the control of the metal-sensing MerR protein, and it is this system that mediates mercury resistance. Mercury resistance depends upon the expression of these genes to import toxic Hg(lI), reduce Hg(II) to the volatile Hg(0) by NADPH, and often additionally to cleave organomercurials to their corresponding hydrocarbon and Hg(II) species. The MerR protein regulates mercury resistance both negatively and positively. As illustrated in Figure 17, MerR in the absence of Hg(II) binds tightly and sequencespecifically to the promoter. In so doing, MerR inhibits binding to the promoter by RNA polymerase. When Hg^{2+} is added at low concentrations, the metal ion binds specifically and with high affinity to the DNA-bound MerR, and causes a DNA conformational change detectable by using other metal reagents as conformational probes. This conformational change now facilitates the binding of RNA polymerase and hence activates expression of the gene family.

What are the structural requirements in the metal-binding site? Certainly one requirement is Hg(II) specificity, so that other metal ions will not also trigger transcriptional activation. Another is high metal-binding affinity to protect the DNA from direct coordination of the Hg(II). The MerR protein is dimeric, and contains four cysteine residues per monomer. Site-directed mutagenesis studies have indicated that three of these four cysteine residues are needed for Hg(II) binding, and EXAFS studies have been consistent with tricoordinate ligation, clearly a well-designed system for Hg(II) specificity. Perhaps even more interesting, the site-directed mutagenesis studies on heterodimers (a mixture of mutant and wild-type monomers) have indicated that the coordinated Hg(II) bridges the dimer, ligating two cysteines of one monomer and one cysteine of the other. This scheme may provide the basis also for the kinetic lability needed in a rapidly responsive cellular system.

Model systems are also being constructed to explore metal modulation of DNA binding. One system involves the assembly of two dipeptides linked by a central acyclic metal-binding polyether ligand, with $Fe(EDTA)^{2-}$ tethered to one end to mark site-specific binding. In the presence of alkaline earth cations, which induce a conformational change that generates a central macrocycle, the linked peptides become oriented to promote sequence-specific binding in the

minor groove. In the absence of the alkaline earth ion, no site-specific binding, or cleavage of DNA, is evident. One might consider this system as a simple, first-order synthetic model for the metalloregulatory proteins.

MerR is clearly only one natural metalloregulatory system. Other metal ions bind regulatory factors to mediate the regulation of gene expression in a metalspecific manner. Two examples include the Fe(II)-binding Fur protein from enteric bacteria and the copper-binding protein ACEl/CUP2 from S. *cerevisiae.* Both copper and iron are essential trace elements for which high concentrations are toxic; for nucleic acids this toxicity is certainly the result of redox-mediated strand damage. Other metal-specific regulatory systems are surely present as well. Both the MerR and the synthetic system may exemplify how these various systems function, how Nature might construct a ligand system to facilitate toxicmetal- specific binding in the presence of DNA that then alters or triggers how other moieties bind and access the nucleic acid.



A model for MerR metalloregulation.[90a] In the absence of MerR, RNA polymerase binds and transcribes the MerR promoter. In the presence of MerR, the preferential binding of MerR to the promoter is observed that inhibits transcription by the polymerase. The addition of Hg^{2+} then leads to a conformational change that promotes binding of the polymerase, substantially increasing transcription. Reproduced with permission from Reference 90a.

FIGURE 17

A Pharmaceutical Role

With the exception of cisplatin , most pharmaceuticals currently being used as DNA-binding agents were first isolated as natural products from bacteria, fungi, plants, or other organisms. For the most part they represent complex organic moieties, including peptide and/or saccharide functionalities, and often a unique functionality, such as the ene-diyne in calichimycin. These natural products bind DNA quite avidly, through intercalation, groove binding, or a mixture thereof. Often the efficacy of these antitumor antibiotics stems from subsequent alkylation or DNA strand-cleavage reactions that damage the DNA. Among the various natural products used clinically as antitumor antibiotics are bleomycins, a family of glycopeptide-derived species isolated from cultures of *Streptomyces.*

The structure of bleomycin A_2 is shown schematically in Figure 18.

The molecular mode of action of these species clearly involves binding to DNA and the promotion of single-stranded cleavage at GT and GC sequences. Importantly, as demonstrated by Horwitz, Peisach, and coworkers, this DNA cleavage requires the presence of Fe(II) and oxygen. Thus, one might consider Fe-bleomycin as a naturally occurring inorganic pharmaceutical. What is the role of the metal ion in these reactions? As one might imagine, based upon our earlier discussions of metal-promoted DNA cleavage, the iron center is essential for the oxidative cleavage of the strand through reaction with the sugar moiety. The reaction of Fe(II)-bleomycin can, however, clearly be distinguished from the $Fe(EDTA)^{2-}$ reactions discussed earlier in that here no diffusible intermediate appears to be involved. Instead of generating hydroxyl radicals, the Fe center must be positioned near the sugar-phosphate backbone and activated in some fashion to promote strand scission *directly.*

Despite extensive studies, in fact little is known about how Fe(II)-bleomycin is oriented on the DNA. Indeed, the coordination about the metal is the subject of some debate. The structure of Cu(II)-P-3A, a metallobleomycin derivative, is also shown in Figure 18. On the basis

of this structure and substantive spectroscopic studies on Fe-bleomycin itself, it is likely that, as with Cu(II), in the Fe(II)-bleomycin complex the metal coordinates the β-hydroxyimidazole nitrogen, the secondary amine of β-aminoalanine, and the Nl nitrogen of the pyrimidine. Whether in addition the primary amines of the amino alanine and of the histidine coordinate the metal is still not settled. Possibly bithiazole coordination or some coordination of the sugar moieties is involved. Nonetheless, given five different coordination sites to the bleomycin, the sixth axial site is available for direct coordination of dioxygen. How is this Fe-0_2 complex oriented on the DNA? It is likely that at least in part the complex binds against the minor groove of the helix. There is some evidence that suggests that the bithiazole moiety intercalates in the helix. It is now becoming clear, however, that the structure of the metal complex itself, its three-dimensional shape, rather than simply the tethered bithiazole or saccharide, is needed for the sequence selectivity associated with its mode of action.

Although the coordination and orientation of the metal complex are still not understood, extensive studies have been conducted concerning the remarkable chemistry of this species. The overall mechanism of action is described in Figure 18. In the presence of oxygen, the Fe(Il) O_2 species is formed and is likely rapidly converted to a ferric superoxide species. The one-electron reduction of this species, using either an organic reductant or another equivalent of Fe(II)-bleomycin, leads formally to an Fe(III)-peroxide, which then undergoes O-O bond scission to form what has been termed "activated bleomycin."

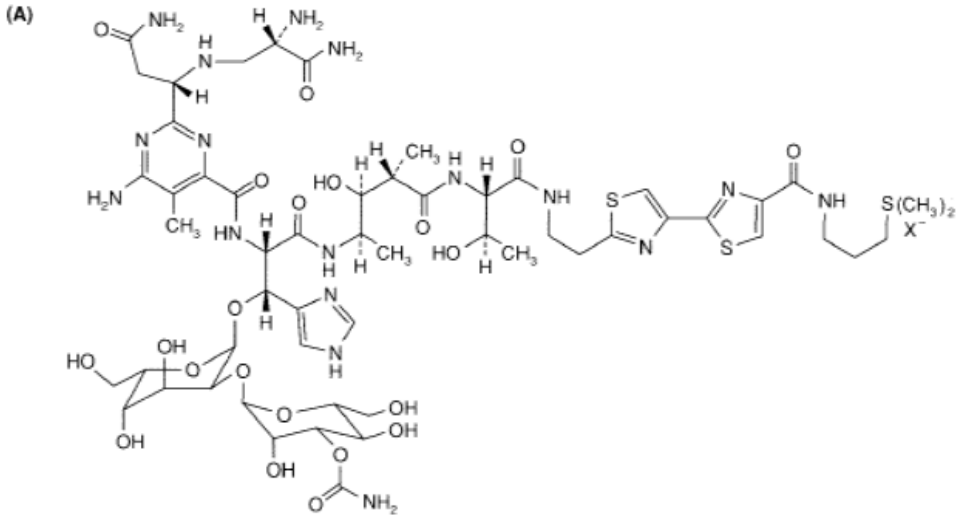
This species might be best described as Fe(V) = 0 (or $[Fe0]^{3+}$). This species is comparable in many respects to activated cytochrome P-450 or perhaps even more closely to the Fe center in chloroperoxidase. Like these systems, activated bleomycin can also epoxidize olefins and can generally function as an oxo transferase. In contrast to these systems, Fe-bleomycin clearly lacks a heme. How this species can easily shuttle electrons in and out, forming and reacting through a high-valent intermediate, without either the porphyrin sink or another metal linked in some fashion, is difficult to understand. In fact, understanding this process, even independently of our fascination with how the reaction is exploited on a DNA helix, forms the focus of a substantial effort of bioinorganic chemists today. What has been elucidated in great detail is the reaction of activated bleomycin with DNA. It has been established that the activated species promotes hydrogen abstraction of the C4'-H atom, which is positioned in the minor groove of the helix (Figure 18). Addition of another equivalent of dioxgen to this C4'-radical

leads to degradation of the sugar to form a 5'-phosphate, a 3'-phosphoglycolate, and free base propenal. Alternatively, oxidation of the C4'-radical followed by hydroxylation in the absence of oxygen yields, after treatment with base, a 5'-phosphate, an oxidized sugar phosphate, and free base.

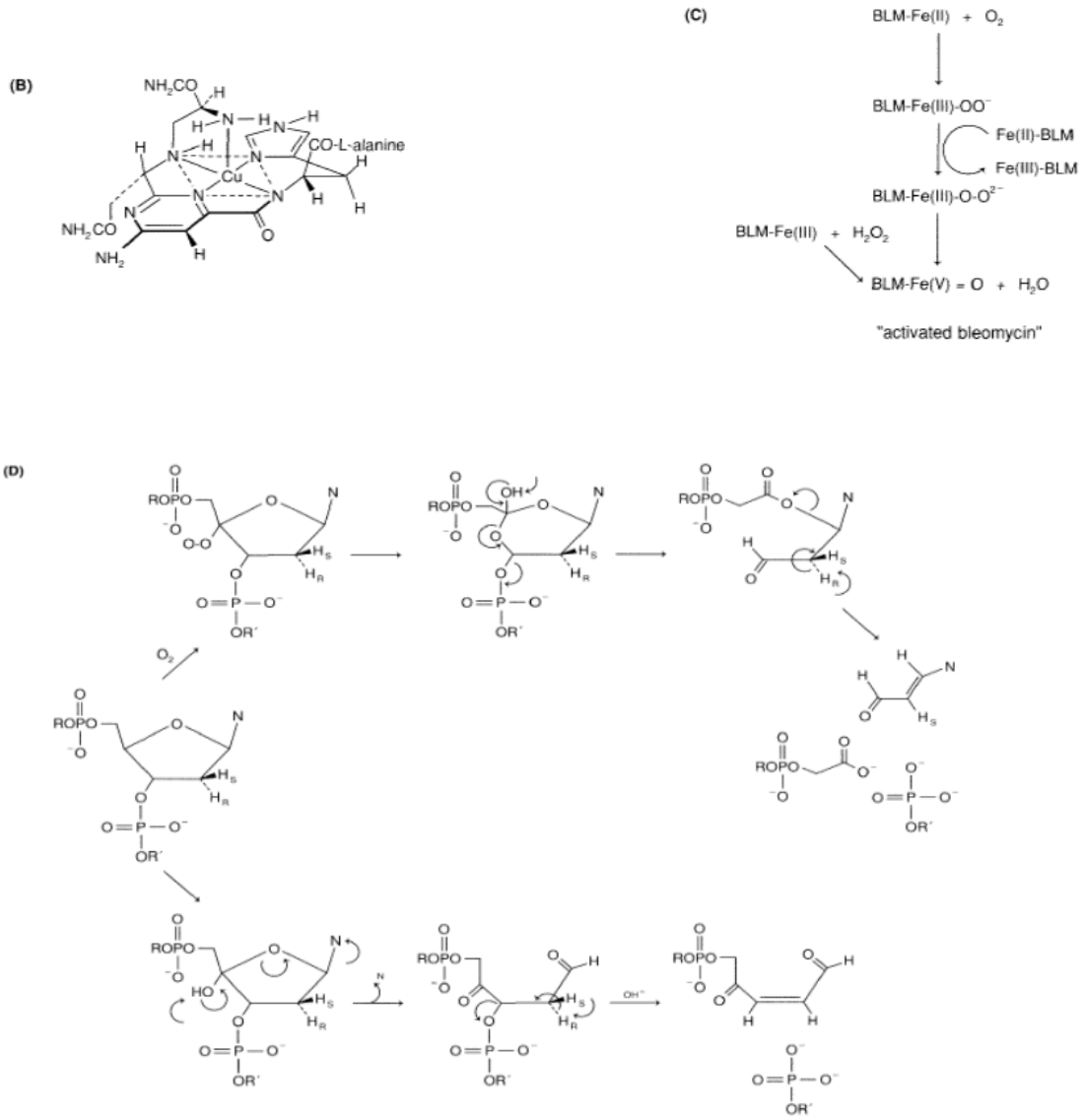
Other metals such as copper and cobalt can also activate bleomycins, although their mechanistic pathways for strand scission are clearly different from that of Fe(II)-bleomycin. Whether other natural products that bind DNA also chelate metal ions and exploit them for oxidative strand cleavage is not known, but several systems provide hints that they do. Furthermore, such a fact would not be surprising given our understanding of the utility of metal ions in promoting this chemistry. An even more detailed understanding of this chemistry might lead to the development of second-generation synthetic transition-metal pharmaceuticals that specifically and efficiently target and cleave DNA sites.

A Catalytic Role

In addition to serving structural and modulating roles in proteins which bind nucleic acids, metal ions also appear to be essential to the functioning of various complex enzymes that act on nucleic acids. At this stage our understanding of the participation of the metal ion in the catalytic chemistry of these enzymes is somewhat sketchy, and we are relying more on our current understanding of the possible roles where



bleomycin



(A) The structure of bleomycin A_2. (B) The crystallographically determined structure[98] of a copper derivative of bleomycin, P3A. (C) A scheme to generate "activated bleomycin." (D) The proposed mechanism of action of activated Fe-bleomycin.

Figure 18

metal ions may prove advantageous. These remain areas of biochemical focus where the inorganic chemist could make a major contribution.

For example, zinc ion appears to be essential to the functioning of both RNA polymerases and DNA topoisomerases. These multisubunit enzymes perform quite complex tasks. RNA polymerase must bind site-specifically to its DNA template, bind its nucleotide and primer substrates, and form a new phosphodiester bond in elongating the growing RNA. Two zinc ions appear to be involved. One may be involved in orienting the nucleotide substrate, and the other structurally in template recognition. It would not be surprising, however-indeed, it might be advantageous-if one or both metal ions also participated in the polymerization step. Our mechanistic understanding of how topoisomerases function is even more cursory. These complex enzymes bind supercoiled DNA, sequentially break one strand through hydrolytic chemistry, move the strand around the other (releasing one tertiary turn), and relegate the strand. Again, the zinc ion might participate in the hydrolytic chemistry, the ligation step, or both; alternatively, the metal might again serve a structural role in recognition of the site of reaction. We do have some understanding of the role of metal ions in several endonucleases and exonucleases. As discussed in Section II.C, metal ions may effectively promote phosphodiester hydrolysis either by serving as a Lewis acid or by delivering a coordinated nucleophile. Staphylococcal nuclease is an extracellular nuclease of *Staphylococcus aureus* that can hydrolyze both DNA and RNA in the presence of Ca^{2+}. The preference of the enzyme is for singlestranded DNA, in which it attacks the 5'-position of the phosphodiester linkage, cleaving the 5'-P-O bond to yield a 5'-hydroxyl and 3'-phosphate terminus.

Ca^{2+} ions are added as cofactors and are strictly required for activity. The structure of staphylococcal nuclease, determined by x-ray crystallography and crystallized in the presence of Ca^{2+} and the enzyme competitive inhibitor pdTp, as well as subsequent NMR and EPR studies on mutant enzymes using Mn^{2+}as a substitute for the Ca^{2+} ion, have provided the basis for a detailed structural analysis of the mechanism of this enzyme. In this phosphodiester hydrolysis, the metal ion appears to function primarily as an electrophilic catalyst, polarizing the P-O bond, and stabilizing through its positive charge the evolving negative charge on the phosphorus in the transition state. The base is thought here not to be directly coordinated to the metal; instead, action of a general base is invoked.

Metal ions also participate in the functioning of other nucleases, although the structural details of their participation are not nearly as established as those for staphylococcal nuclease. DNAse I also requires Ca^{2+} for its catalytic activity. S1endonuclease, mung bean nuclease, and

Physarum polycephalem nuclease require zinc ion either as cofactors or intrinsically for nuclease activity, and the restriction enzyme EcoRI may also require intrinsically bound zinc ion. In terms of how the zinc ion might function in these enzymes, one can look both to staphylococcal nuclease and to bacterial alkaline phosphatase for some illustrations. One would expect that this metal ion could serve both as an electrophilic catalyst and also in the delivery of a zinc-coordinated hydroxide, as it does in alkaline phosphatase, directly attacking the phosphate ester. More work needs to be done to establish the mechanisms by which zinc ion promotes phosphodiester hydrolysis in these enzymatic systems.

Probably most intriguing and mysterious at this stage is the metal participation in the very complex DNA-repair enzyme endonuclease III from *E. coli* (similar enzymes have also been isolated from eukaryotic sources). This enzyme is involved in the repair of DNA damaged by oxidizing agents and UV irradiation, and acts through an N-glycosylase activity to remove the damaged base, and through an apurinic/apyrimidinic endonuclease activity to cleave the phosphodiester bond adjacent to the damaged site. Although more complex in terms of recognition characteristics, this enzyme functions in hydrolyzing the DNA phosphodiester backbone. What is so intriguing about this enzyme is that it contains a 4Fe-4S cluster that is essential for its activity. We think generally that Fe-S clusters best serve as electron-transfer agents. In the context of this repair enzyme, the cluster may be carrying out both an oxidation and a reduction, to effect hydrolysis, or alternatively perhaps a completely new function for this metal cluster will emerge. (Fe-S clusters may represent yet another structural motif for DNA-binding proteins and one which has the potential for regulation by iron concentration.) Currently the basic biochemical and spectroscopic characterization of the enzyme is being carried out. Understandingthis very novel interaction of a metal center and nucleic acid will require some new ideas, and certainly represents one new challenge for the bioinorganic chemist.

Reference:

1. BioInorganic Chemistry, Chapter 8, Metal / Nulcleic Acid Interactions, University Science Books, Mill Valley, California.