Nickel-Dependent Oxidative Cross-Linking of a Protein†

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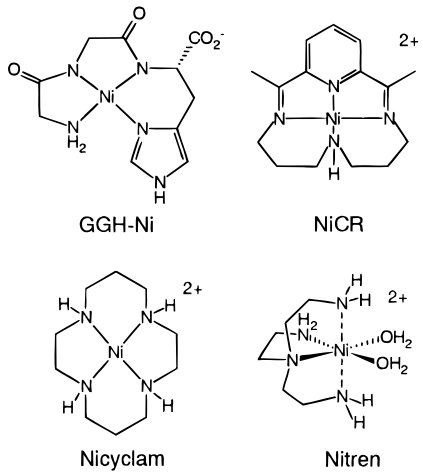
A model protein, ribonuclease A (bovine pancreas), was examined for its ability to coordinate Ni^{2+} and promote selective oxidation. In the presence of a peracid such as monopersulfate, HSO_5^-, nickel induced the monomeric RNase A to form dimers, trimers, tetramers, and higher oligomers without producing fragmentation of the polypeptide backbone. Co^{2+} and to a lesser extent Cu^{2+} exhibited similar activity. The nickel-dependent reaction appeared to result from a specific association between the protein and Ni^{2+} that allowed for transient and in situ oxidation of the bound nickel to yield intermolecular tyrosine−tyrosine cross-links. Macrocylic nickel complexes that had previously been shown to promote guanine oxidation were unable to mimic the activity of the free metal salt. Amino acid analysis of the protein dimer confirmed the expected consumption of one tyrosine per polypeptide and formation of dityrosine. The presence of excess tyrosine efficiently inhibited formation of the protein dimer and produced instead a ribonuclease−tyrosine cross-link. In contrast, high concentrations of the hydroxyl radical quenching agent mannitol only partially inhibited ribonuclease dimerization. The polypeptide-mediated activation of nickel and its subsequent reactivity mimic a process that could contribute to the adverse effects of nickel in vivo.

The chemical and biological properties of nickel are highly dependent on its surrounding ligands. Both the coordination geometry and oxidation−reduction potential are significantly altered when simple salts forming Ni-$(H_2O)_6^{2+}$ in aqueous solution are chelated by strong donor ligands. Crystalline, amorphous, and metallic nickel have been shown to produce tumors in animals and may share a common mechanism for this involving intracellular Ni(II) despite differences in their initial exposure, uptake, and solubilization (for review, see refs *1*−*3*). This transition metal ion stongly associates with amino acids, peptides, proteins, and nucleic acids. Such chelation can be extremely stable and exhibit dissociation constants with the tripeptides, GGH, GGG, and DAH, or serum albumin on the order of 10^{-10} M^{-1} (*4*, *5*). Some deleterious effects of nickel may result solely from coordination, but most are likely related to the oxidative activity of these complexes.

Under physiological conditions, simple nickel salts exhibit little intrinsic oxidation or reduction chemistry particularly in contrast to iron, copper, and cobalt (*2*, *6*). However, after nickel binds to the backbone of peptides and proteins, it becomes quite easily oxidized by O_2, $O_2^{\bullet-}$, and H_2O_2 (*7*, *8*). GGH−Ni(II), glutathione−Ni(II), and other oligopeptide complexes of Ni(II) have been shown to promote lipid and protein oxidation in the presence of peroxides to a much greater extent than either free Ni^{2+} or ligand alone (*9*, *10*). Similarly, GGGG−Ni(II) induced polymerization of histones (*11*), and histidine−Ni facilitated oxidation of deoxyguanosine (*12*).



The ability to control the redox chemistry of nickel by appropriate ligand design has also allowed for development of numerous reagents applicable in vitro. Our laboratories have characterized nucleic acid structure with a series of macrocyclic nickel complexes including (2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17),2,11,13,15-pentaenato) nickel(II) (NiCR)[1] and 1,4,8,-11-tetraazacyclotetradecane nickel(II) (Nicyclam) that oxidize guanine residues according to the accessibility of the guanine N7 atom (*13*, *14*). Use of an alternative ligand promoted covalent bond formation between the nickel complex and the same accessible guanines (*15*). These examples all relied on a peracid as the terminal

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[1] Abbreviations: CAPS, 3-cyclohexylamino-1-propanesulfonate; 2D-COSY, two-dimensional correlated spectroscopy; MCPBA, *meta*-chloroperbenzoate; MMPP, magnesium monoperoxyphthalate; NiCR, (2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17),2,11,13,15-pentaenato)nickel(II); PVDF, poly(vinylidene difluoride); TSP, 3-(trimethylsilyl)propionate; tren, tris(2-aminoethyl)amine nickel(II); cyclam, 1,4,8,11-tetraazacyclotetradecane.

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oxidant, but related derivatives have been investigated that activate O_2 for DNA modification (*16*). Others have demonstrated the use of a bleomycin−nickel complex for similar identification of guanine (*17, 18*). Nickel−peptide complexes have additionally been adapted for sequence and structure specific probes of DNA (*19*−*22*). In each case, the nickel-based reagents benefit from the formation of a non-diffusible oxidant.

The redox chemistry of nickel−peptide complexes has concurrently been applied to mapping protein−drug (*23*) and protein−protein interactions (*24*). Likewise, other transition metal complexes have been employed to characterize protein structure in analogy to their use in the field of nucleic acids. For example, the nonspecific cleavage of a protein by EDTA−Fe(II) (*25*) could be localized by tethering this complex to a protein (*26*−*29*) or biotin (*30*). A comparable study made use of the native recognition of tetracycline−Fe(II) for specific protein cleavage (*31*). Sequence and structure specific hydrolysis of proteins may also soon be possible as a consequence of the exciting observation that Pd(II) could selectively hydrolyze dipeptides containing *N*-acetyl-His-X (*32*). This latter approach may ultimately allow for polypeptide fragmentation at accessible sites adjacent to histidine residues with a simple metal complex thereby eliminating the need for specialized ligands or tethers. We pursued a similar strategy for protein oxidation based on the intrinsic ability of free Ni^{2+} to bind a peptide backbone spontaneously (*5*) and create a metal−peptide site that would be sensitive to oxidants (*8*). Flexible loop and coil regions were the expected targets since Ni(II) would most easily be accommodated in these regions. Initial studies described below demonstrate that nickel readily bound to the monomeric protein RNase A and led to its oxidative cross-linking to form multimeric products. An alternative process, protein fragmentation, was not observed and therefore not competitive with amino acid side chain oxidation.

Materials and Methods

Caution. Perchlorate salts of metal complexes with organic ligands are potentially explosive and should be handled carefully to avoid mechanical agitation. These salts should be prepared in small quantities only.

Materials. Ribonuclease A from bovine pancreas Type III-A was purchased from Sigma (St. Louis, MO) and used directly in all experiments. Molecular weight standards for electrophoresis under denaturing conditions were obtained from Bio-Rad (Hercules, CA) and contained *Escherichia coli* β-galactosidase (97 kDa), bovine serum albumin (66 kDa), egg white ovalbumin (42 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). All buffer solutions were made with distilled, deionized, and filtered water (Nanopure, Sybron/Barnstead, Dubuque, IA). Reagents and buffers were purchased from Fisher Scientific (Pittsburgh, PA), Aldrich (Milwaukee, WI), and Sigma in the highest purity available and used without further purification. Metal salts had a grade of Certified (American Chemical Society). Diaqua [tris-(2-aminoethyl)amine nickel(II)] acetate (Ni(II)-tren) and [1,4,8,-11-tetraazacyclotetradecane nickel(II)] perchlorate (Ni(II)-cyclam) were synthesized by Dr. James Muller (University of Utah) according to published procedures (*33*).

Standard Conditions for Oxidative Cross-Linking of RNase A. Reaction mixtures (50 μL) of RNase (400 μM) and $Ni(OAc)_2$ (50 μM) were pre-incubated in 10 mM sodium borate (pH 10) for 30 min under ambient temperature. Cross-linking was initiated by addition of $KHSO_5$ (800 μM) and quenched after 30 min by adding an equal volume of a standard denaturing buffer for electrophoresis (4% SDS, 25% glycerol, 140 mM β-mercaptoethanol, 150 mM Tris (pH 6.8), and 0.1% bromophenol blue). Samples were then heated to 100 °C for 5 min and analyzed by SDS−polyacrylamide gel electrophoresis using a Laemmili discontinuous system with 5% stacking and 15% resolving gels (*34*). Proteins were stained by Coomassie Blue R-250. Reactions were quantified by densitometric analysis using an Enprotech scanner and software (Natick, MA).

RNase was alternatively cross-linked by horseradish peroxidase (*35*) under the same conditions described above except 0.08 μM peroxidase was used in place of the nickel salt, and 800 μM H_2O_2 was used in place of the $KHSO_5$. The reaction was quenched, denatured, and analyzed as described above.

Amino Acid Analysis of RNase A and Its Derivatives after Separation by Gel Electrophoresis. Protein fractions were transferred from polyacrylamide gels to poly(vinylidene difluoride) (PVDF) membranes (Bio-Rad) by electroblotting. The regions of the membrane containing protein were excised and submitted for amino acid analysis (Picotag, Millipore Corp., Milford, MA) to the Center for Analysis and Synthesis of Macromolecules at Stony Brook. A standard of 3,3′-dityrosine was synthesized according to the procedure below.

Synthesis and Model Formation of 3,3′-Dityrosine. Tyrosine (13 mM), $Ni(OAc)_2$ (26 mM) and 6 mM $KHSO_5$ were combined in 10 mM borate buffer (pH 10) and mixed at 22 °C for 30 min. Reaction was followed by removing and filtering (Acrodisc, Fisher Scientific) small aliquots and then measuring the characteristic fluorescence of this dityrosine isomer (*36*). The desired product was obtained after isocratic elution (50 mM triethylammonium acetate, pH 6) through reverse phase HPLC (C-18 column) in an unoptimized yield of 11% based on the limiting reagent, $KHSO_5$. ^{1}H-NMR and 2D-COSY NMR (AMX-600) of the product are consistent with 3,3′-dimerization. ^{1}H-NMR (0.1 M TSP, pH 11) δ 2.78 (dd, J = 15, 5 Hz, 1H, $H_{\beta 1'}$), 2.8 (dd, J = 13, 5 Hz, 1H, $H_{\beta 1}$), 3.02 (dd, J = 13, 5 Hz, 1H, $H_{\beta 2'}$), 3.14 (dd, J = 13, 5 Hz, 1H, $H_{\beta 2}$), 3.5 (dd, J = 7.8, 5 Hz, 1H, $H_{\alpha'}$), 3.56 (dd, J = 7.8, 5 Hz, 1H, H_{α}), 6.84 (d, J = 8.1 Hz, 1H, H-6′), 7.09 (d, J = 8.1 Hz, 1H, H-6), 7.13 (d, J = 8.3 Hz, 1H, H-5′), 7.27 (d, J = 8.2 Hz, 1H, H-5), 7.3 (s, 1H, H-2′), 7.4 (s, 1H, H-2).

Results

Ni(II) Promoted Oxidative Cross-Linking of Monomeric RNase A in the Presence of $KHSO_5$. Incubation of RNase A (13.7 kDa) in the presence of Ni^{2+} and HSO_5^- generated high molecular weight products corresponding to RNase dimer, trimer, and higher oligomers (Figure 1A, lane 2). Both Ni^{2+} and HSO_5^- were required for this process, and addition of a chelator such as EDTA (4 mM) completely inhibited cross-linking. No competing fragmention of RNase was evident under any conditions, and therefore, oxidative modification of the polypeptide backbone was unlikely under these conditions. The dimerization process was mimicked by the action of horseradish peroxidase and H_2O_2 (Figure 1A, lane 3), which is known to couple proteins via formation of dityrosine (*35, 36*). Consistent with this model process, the nickel-dependent reaction was similarly controlled by pH, and an optimal yield was observed at pH 10. Accordingly, all further experiments were performed at this pH using borate buffer. Although borate may selectively complex dityrosyl residues (*37*), this buffer was not necessary for RNase dimerization and could be replaced with 3-cyclohexylamino-1-propanesulfonate (CAPS, 10 mM).

Under standard conditions, the oxidant HSO_5^- was the limiting reagent and reaction was complete prior to quenching (30 min). Use of additional HSO_5^- (>800 μM) resulted in further consumption and extensive oligomerization of the monomeric enzyme to yield high molecular weight products. Nickel was not consumed in this

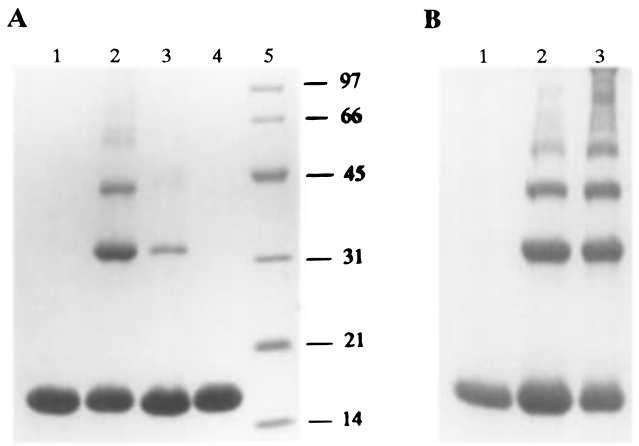


Figure 1. Oxidative cross-linking of RNase A in the presence of $Ni(OAc)_2$ and $KHSO_5$. (A) RNase A (lane 1) was alternatively treated with $Ni(OAc)_2/KHSO_5$ (lane 2) or horseradish peroxidase/H_2O_2 (lane 3) under standard reaction conditions described in Materials and Methods. RNase A was also treated with $KHSO_5$ in the absence of nickel (lane 4). Samples were analyzed by SDS−polyacrylamide gel electrophoresis, stained with Coomassie Blue, and compared to molecular weight markers (lane 5). (B) RNase (400 μM, lane 1) was incubated with $Ni(OAc)_2$ (50 μM) in 10 mM borate buffer pH 10 (lane 2) or 10 mM potassium phosphate buffer pH 7 (lane 3) for 30 min, dialyzed against water (3 × 5 h), and readjusted to pH 10 with 10 mM borate. Reaction was then initiated with $KHSO_5$ (800 μM) and analyzed under standard conditions.

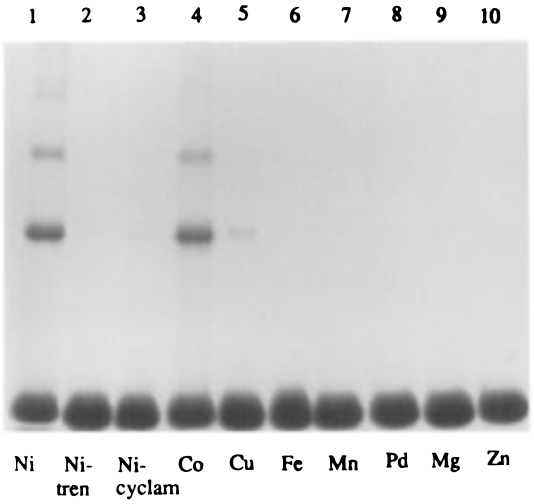


Figure 2. Metal dependence of RNase A oxidative cross-linking. RNase A (400 μM) and various metal salts (50 μM) were combined under standard conditions prior to addition of $KHSO_5$ (800 μM). Samples were incubated for 30 min under ambient conditions and then analyzed by SDS−polyacrylamide gel electrophoresis. Lane 1, $Ni(OAc)_2$; lane 2, Ni(II)tren and 4 mM tren, the free macrocyclic ligand; lane 3, Ni(II)cyclam and 4 mM cyclam, the free macrocyclic ligand; lane 4, $Co(OAc)_2$; lane 5, $Cu(OAc)_2$; lane 6, $Fe(NH_4)_2(SO_4)_2$; lane 7, $Mn(OAc)_2$; lane 8, $PdCl_2$; lane 9, $Mg(OH)_2$; lane 10, $Zn(NO_3)_2$.

process and appeared to act catalytically. Although it bound tightly to RNase (see below), it remained kinetically labile enough to dissociate from cross-linked protein and reassociate with the native, monomeric protein. Quantifying the reaction illustrated in Figure 1B (lane 3) suggested that less than 50 μM $Ni(OAc)_2$ promoted consumption of approximately 250 μM RNase, the result of more than 2.5 turnovers.

Strong Association between Nickel and RNase A. If an association between Ni^{2+} and RNase were required for reaction as predicted, then lack of reaction at pH 7 might have been a function of metal binding or protein oxidation. RNase affinity for Ni^{2+} was qualitatively examined by incubating this protein with $Ni(OAc)_2$ at pH 7 (10 mM potassium phosphate) and pH 10 (10 mM sodium borate), dialyzing against water, and then assaying for bound Ni(II) by its oxidation of RNase after subsequent addition of HSO_5^- at pH 10. Sufficient Ni(II) resisted dialysis at pH 7 to promote protein cross-linking equivalent to that of samples dialyzed at pH 10 (Figure 1B). This suggests that Ni(II) binds strongly to RNase in a manner independent of pH 7−10. As expected, alternative dialysis in the presence of EDTA generated RNase samples that yielded no dimerization after addition of HSO_5^-.

Metal Dependence of RNase Cross-Linking. A series of common transition metals were surveyed for an equivalent ability to induce protein cross-linking in the presence of HSO_5^-. This was necessary in part to verify that a contaminating metal was not responsible for the activity ascribed to nickel. Even trace metals had the potential to dominate reaction since Ni^{2+} is not often associated with oxidation chemistry under aqueous conditions. In contrast to many expectations, $Ni(OAc)_2$ was one of the two most active metal salts tested for RNase cross-linking (Figure 2). Cobalt and copper were also competent, but only cobalt exhibited an efficiency similar to nickel.

Initial studies had suggested that a limited number of macrocyclic Ni(II) complexes forming square planar and octahedral geometries were also capable of dimerizing RNase albeit in much lower yields than $Ni(OAc)_2$. However, no such activity was detected when additional free ligand (4 mM) was added to minimize dissociation of nickel from the macrocyclic complex (Figure 2, lanes 2 and 3). After the Kodadek laboratory reported the use of GGH−Ni for cross-linking multimeric proteins (*24*), we tested this nickel peptide complex with RNase under their published conditions [pH 7, 10 μM protein, 100 μM GGH−Ni, and 100 μM magnesium monoperoxyphthalate (MMPP), a peracid analogous to HSO_5^-]. Interestingly, this also led to formation of RNase dimer, trimer, and tetramer (data not shown). The mechanism of this latter reaction may differ from that described here since none of the nickel species presented in Figure 2 exhibited the activity of GGH−Ni at pH 7.

Oxidant Dependence of RNase Cross-Linking. Most biochemical applications of nickel peptide and macrocyclic complexes have used the peracids HSO_5^- and MMPP as terminal oxidants. Such reagents were also most effective in supporting nickel-dependent cross-linking of RNase. The strongest oxidants, HSO_5^-, MMPP, and *meta*-chloroperbenzoate (MCPBA), provided the highest yield of oligomeric RNase (Figure 3). Persulfate, $S_2O_8^{2-}$, also supported efficient cross-linking (Figure 3, lane 2). In contrast, peroxides were inactive (Figure 3, lanes 6−8).

Quenching by Free Radical Trapping Agents. Once Ni(II) becomes redox active through coordination to a peptide backbone, a variety of oxidative processes may be stimulated. In the presence of H_2O_2, GGH−Ni promoted formation of the diffusible hydroxyl radical that led to oxidative degradation of tryptophan and formation of dityrosine in bovine serum albumin and lactate dehydrogenase (*10*). This reaction was inhibited by standard radical quenching agents including ethanol, butanol, and dimethyl sulfoxide. To examine the possible role of hydroxyl radical or other activated oxygen species in the

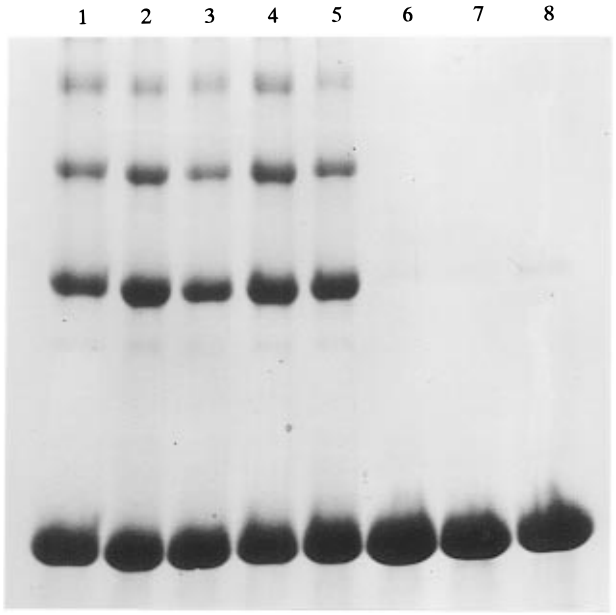


Figure 3. Oxidant dependence of RNase A cross-linking. The nature of the oxidant required for the nickel-mediated reaction of RNase was examined by substituting $KHSO_5$ (lane 1) with equimolar concentrations (800 μM) of $K_2S_2O_8$ (lane 2), MCPBA (lane 3), MMPP (lane 4), peracetate (lane 5), hydrogen peroxide (lane 6), hydrogen peroxide/ascorbate (lane 7), and *tert*-butyl hydroperoxide (lane 8).

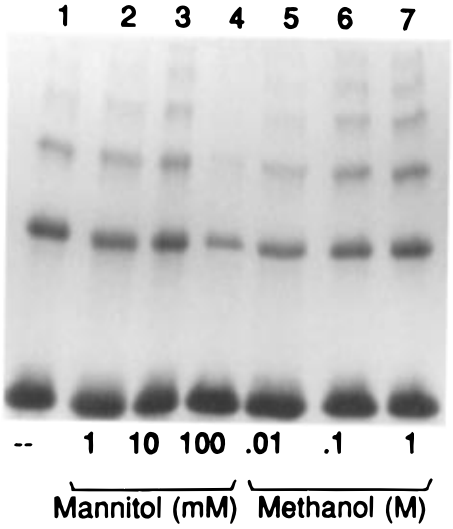


Figure 4. Effect of hydroxyl radical scavengers on the oxidative cross-linking of RNase A. $Ni(OAc)_2$, oxidant, and RNase were reacted under standard conditions (lane 1) and in the added presence of mannitol or methanol.

modification of RNase, the nickel-dependent oxidation was repeated in the presence of 1−100 mM mannitol and 10−1000 mM methanol (Figure 4). These radical scavengers had little effect on protein dimerization, and only partial inhibition was observed for samples containing the highest concentration of mannitol. Therefore, the reactive intermediates generated by the bound nickel remain either highly localized or relatively unaffected by hydrogen atom donors.

Quenching by Free Amino Acids. Accessible tyrosines on the surface of RNase were obvious candidates for intermolecular coupling, and additional targets of oxidation were histidine, lysine, and arginine (*38*−*40*). Tryptophan might also have been expected to react, but this residue is not present in RNase A. To identify which residues could be responsible for cross-linking, each

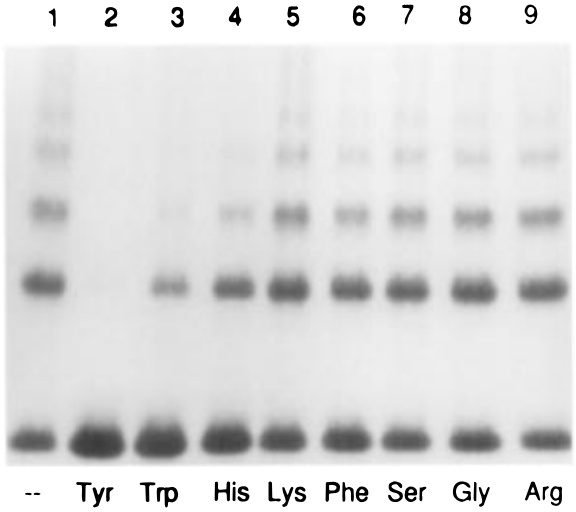


Figure 5. Effect of free amino acids on the oxidative cross-linking of RNase A. $Ni(OAc)_2$, oxidant, and RNase were reacted under standard conditions (lane 1) and in the added presence of the indicated amino acids (800 μM).

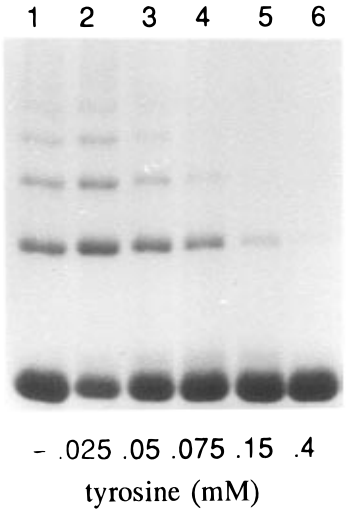


Figure 6. Effect of tyrosine on the oxidative cross-linking of RNase A. $Ni(OAc)_2$, oxidant, and RNase were reacted under standard conditions (lane 1) and in the added presence of tyrosine (lanes 2−6).

amino acid was added to compete with RNase in the nickel-dependent reaction. Tyrosine completely suppressed RNase dimerization (Figure 5, lane 2) when added in a 2-fold excess over protein. Tryptophan and, to a much lesser extent, histidine also inhibited reaction under these conditions, whereas lysine, phenylalanine, serine, glycine, and arginine had no effect on protein oligomerization (Figure 5, lanes 3−9). These latter results suggest that quenching was not simply due to the ability of α-amino acids to bind Ni^{2+} and perhaps sequester it from RNase.

Protein oligomerization was extremely sensitive to free tyrosine. Inhibition of this reaction was evident after addition of tyrosine at concentrations equimolar to $Ni(OAc)_2$ (0.05 mM, Figure 6, lane 3), and quenching was almost complete (82%) when the tyrosine concentration was increased to 0.15 mM (Figure 6, lane 5) but well below the concentration of RNase (0.4 mM) and HSO_5^- (0.8 mM).

Amino Acid Analysis of RNase A and Its Products After Oxidation. Amino acid analysis of RNase was used to ascertain which amino acids were subject to the

Table 1. Amino Acid Analysis of RNase A and Its Derivatives Isolated after Treatment with $Ni(OAc)_2$ and $KHSO_5$

	amino acid residues per polypeptide[a]			
residue	monomer (theoretical)	monomer[b]	dimer	tyrosine-coupled monomer[c]
Dityr	−	≤0.2	0.6	0.6
Tyr	6	6.1	5.1	5.3
Ala	12	11.9	11.7	11.5
Arg	4	4.1	4.3	4.2
Asx	15	15.4	14.9	14.9
Gly	3	3.4	3.5	3.2
Glx	12	12.4	12.4	11.9
His	4	4.0	3.7	3.8
Ile	3	2.4	2.3	2.3
Leu	2	2.5	2.4	2.5
Lys	10	9.5	8.7	9.0
Phe	3	3.1	3.0	3.0
Pro	4	4.6	4.7	4.4
Ser	15	13.6	13.7	12.8
Thr	10	9.6	9.7	8.6
Val	9	9.1	9.1	9.0

[a] RNase A contains four Met and eight Cys that were not measured in these analyses. All standard deviations were less than 5% of the average from three independent determinations. [b] This species represents the monomeric protein isolated after reaction by gel electrophoresis. [c] This derivative was isolated as the monomer after standard nickel-dependent oxidation in the added presence of 16 mM tyrosine.

nickel-dependent oxidation. Both monomeric and dimeric protein were blotted from standard polyacrylamide gels, subjected to exhaustive hydrolysis (6 N HCl, 24 h, 110 °C) and quantified using standard chromatography. These procedures were also repeated for the monomeric protein oxidized in the presence of added tyrosine. The average composition from three independent determinations is summarized in Table 1. To facilitate comparison between samples, stoichiometries are listed per single polypeptide rather than per mole since the RNase dimer contains two polypeptides.

Analysis of the monomeric enzyme recovered after oxidation indicates that few, if any, residues were modified other than those participating in cross-linking. The ratio of amino acids for this protein was close to the theoretical value. Only the concentrations of isoleucine, lysine, threonine, and particularly serine were lower than expected although still typical of past analyses of RNase (for example, see ref *41*). The covalent dimer generated one less tyrosine per polypeptide strand and produced a new material that co-eluted with 3,3′-dityrosine synthesized as described below. The slight excess of dityrosine detected over the expected 0.5 residue/polypeptide (or 1 residue/dimer) may be attributed to the ≤0.2 residue background observed for the monomeric protein recovered after reaction. Analysis of a similar monomeric derivative isolated after treatment with $Ni(OAc)_2$, HSO_5^-, and a large excess of free tyrosine also indicated the loss of a tyrosyl residue. However, a substoichiometric yield of 3,3′-dityrosine was detected which suggested alternative products such as an O-coupled isodityrosyl derivative might have formed (*42*). Finally, the characteristic fluorescence excitation (310 nm) and emission (410 nm) maxima of 3,3′-dityrosyl residues (*36*) was used to confirm its presence in the covalent dimer of RNase and its absence in the products of control reactions containing RNase alone, RNase and $Ni(OAc)_2$, or RNase and HSO_5^-.

Synthesis of 3,3′-Dityrosine As a Standard for Amino Acid Analysis. Tyrosine was oxidatively dimerized under conditions related to those used to modify RNase. Chromatographic analysis detected only the presence of 3,3′-dityrosine and unmodified starting material. Under these conditions, tyrosine coupling was promoted by $Ni(OAc)_2$ and the nickel complexes and strongly inhibited by mannitol (100 mM). In contrast to protein dimerization, this model reaction likely depended on the generation of a diffusible radical.

Discussion

Application of a Simple Nickel Salt. Much of nickel's biological activity is controlled by the endogenous ligands that surround solubilized Ni^{2+} (*1, 2, 6*). Similarly, much of nickel's utility as a biochemical reagent relies on proper ligand design since coordination alone does not guarantee an oxidation chemistry compatible with aqueous conditions. Peptides and proteins provide one of the most significant classes of ligands for consideration both in vitro and in vivo. Nickel has a strong propensity to bind and deprotonate an amide backbone which allows for subsequent reaction with a variety of oxidants. Selective modification of proteins may therefore be anticipated from coordination to compliant peptide sequences. Related processes are likely responsible for stimulating oxidative stress and aging associated with exposure to nickel (*11, 43*−*45*).

The present study demonstrates the ability of a simple nickel salt to bind to a monomeric protein, RNase A, and promote its oligomerization in the presence of a peracid, HSO_5^-. Individual dimer, trimer and tetramers of RNase were evident from electrophoretic analysis of protein oxidation (Figure 1A). Little heterogeneity was apparent in these products, and no protein fragmentation was discernible. These observations presented the first indication that the nickel-dependent oxidation of RNase was therefore selective in its chemistry.

While the use of RNase at high concentration (400 μM) is convenient for the present investigation, the cross-linking remained detectable for reaction mixtures diluted 50-fold [1 μM $Ni(OAc)_2$, 8 μM RNase, 16 μM HSO_5^-]. These alternative conditions more closely mimicked those used to cross-link a series of proteins by an oxidative process mediated by GGH−Ni (*24*). In contrast to the activity of this nickel tripeptide complex, cross-linking induced by the RNase−Ni complex was not observed at pH 7. Nickel appeared to bind spontaneously to RNase at both pH 7 and 10 (Figure 1B), but protein coupling required the higher pH. The partial deprotonation of Y73, Y76, Y92, and Y115 under these conditions (*46, 47*) would assist oxidation by lowering the one-electron potential of tyrosine (*48*).

Protein Oligomerization Is Formed by Intermolecular Coupling of Tyrosine Residues. Formation of 3,3′-dityrosine is a common product of protein oxidation and was first to be considered in the oligomerization of RNase. Dityrosine has been detected in a wide variety of proteins subjected to oxidation by hydroxyl radical, H_2O_2, peroxidase, H_2Cl_6Ir, and ultraviolet light (*36*). This amino acid derivative also occurs naturally in a number of connective tissue proteins (*38*) and serves as a determinant of protein aging (*39*). Already RNase had been shown to dimerize via tyrosine coupling when exposed to γ-irradiation (*49*). In addition, heteroprotein cross-linking by GGH−Ni was similarly thought to involve intermolecular tyrosine−tyrosine bonds (*24*).

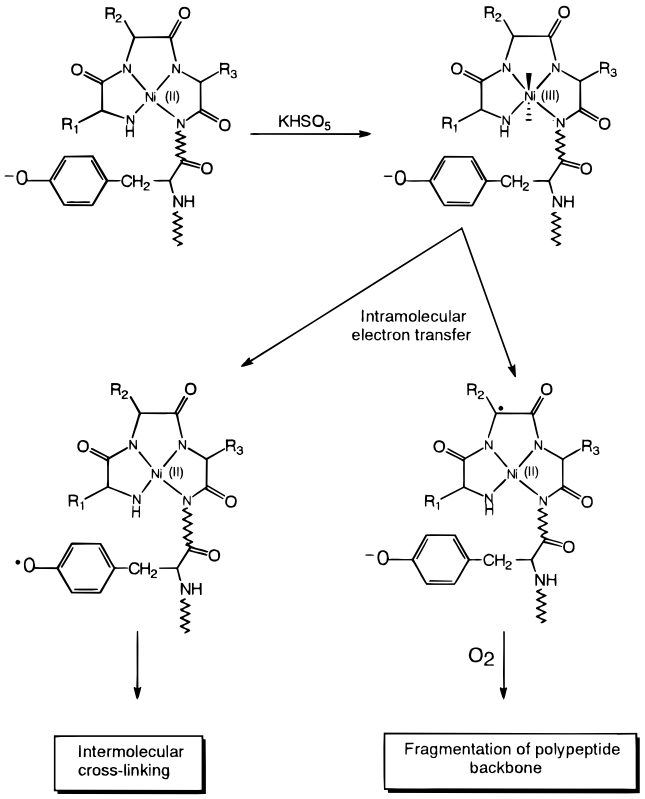
The first experimental evidence implicating tyrosine as the site of oxidition induced by nickel was obtained from a series of competition studies. The oligomerization

of RNase was not inhibited by addition of excess amino acids that might coordinate to Ni^{2+} through the common α-amino acid groups (glycine, serine, phenylalanine, etc., Figure 5) or cationic side chains (arginine, lysine). In contrast, easily oxidized amino acids such as tyrosine and tryptophan effectively quenched protein dimerization (Figure 5).[2] Reaction was most sensitive to tyrosine due to its ability to substitute for RNase as the target for intermolecular coupling to form a protein−tyrosine cross-link (see below). Participation of tyrosine was subsequently confirmed by amino acid analysis. Only one tyrosyl residue per polypeptide was oxidized in the dimeric protein generated by nickel and HSO_5^-, and no other residues were significantly modified when comparing the dimeric vs. monomeric RNase recovered after oxidation (Table 1). Accordingly, tyrosyl modification appeared to dominate intermolecular cross-linking of RNase. Formation of the 3,3′-dityrosyl residue was indicated by both amino acid and fluorometric analysis.

Metal, Oxidant, and Mechanism. Redox active metals ions including Fe^{2+}, Cu^{2+}, and Mn^{2+} all promote oxidative modification of proteins under a variety of conditions (*40*), but these simple ions exhibited little ability to mediate the oxidation of RNase by HSO_5^- (Figure 2). In contrast, the normally unreactive ion Ni^{2+} demonstrated efficient oxidation of RNase while a nickel complex $[Ni(cyclam)]^{2+}$ previously known to induce DNA oxidation remained unreactive (*50*). Such observations are consistent with the ability of the simple nickel ion to bind an accessible region of a polypeptide and promote a unique and localized oxidation. Oligomerization of monomeric RNase by intermolecular reaction of tyrosyl residues was only partially affected by high concentrations of mannitol (Figure 4) and much more sensitive to free tyrosine concentration. Consequently, a diffusible hydroxyl radical is not a plausible intermediate in the nickel-dependent reaction described in this report (*10, 51*). Oxidants typically used to generate hydroxyl radical were also unreactive with RNase−Ni^{2+} (Figure 3). Instead, this protein−nickel complex may act by promoting the transient formation of a Ni(III) intermediate that abstracts an electron from a neighboring tyrosine in analogy to a mechanism previously proposed for cross-linking between a phenolate containing complex of nickel (salen) and DNA (*14, 15*). The peracids and persulfate are all capable of oxidizing the proposed complex from RNase−Ni(II) to RNase−Ni(III) (*52, 53*). In the absence of readily oxidized side chains, the Ni(III) intermediate might alternatively abstract a hydrogen from the polypeptide backbone ultimately promoting protein fragmentation, a process not observed with RNase (see Scheme 1).

The low activity of Cu^{2+} (Figure 2) may result from a mechanism equivalent to, but less effective than, that of nickel. Copper(II) has a similar high affinity for binding peptides and proteins, and the resulting complexes stabilize the related formation of Cu(III) (*54*). In contrast, the Co^{2+} reaction may not require prior binding to the protein. Only Co^{2+} readily generates a diffusible sulfate radical anion from HSO_5^- (*55, 56*). This radical has previously been shown to produce dityrosine and other oxidized products when generated in the presence of albumin (*57*). Quenching studies have now begun to examine the Co^{2+}-dependent reaction of RNase A.

Scheme 1



The terminal oxidant of this investigation, HSO_5^-, was initially chosen due to its ease of handling and successful application to the field of nucleic acids (*14, 58*). However, the cobalt reaction highlights the additional relevance of this oxidant in biology. Cobalt generates sulfate radical anion from sulfite, SO_3^-, and O_2 via intermediate formation of HSO_5^- (*59, 60*). Therefore, Co^{2+} mediates both the production and consumption of this peracid. Our study then describes a process and type of protein modification that could result from natural exposure to cobalt and the common food preservative sulfite. Similarly, a wide variety of nickel complexes utilize HSO_5^- as a terminal oxidant for biopolymer modification, and certain of these complexes will also generate HSO_5^- from sulfite and O_2 (*61*). The nickel-dependent reaction of this report consequently mimicks one of the processes that may contribute to the toxicity and carcinogenicity of this metal.

Association between Nickel and RNase. An important remaining question is where and how does Ni^{2+} bind RNase to promote oxidation. This is the subject of continuing investigations which are aided by numerous peptide studies (see for example refs *8*, *62*, and *63*). Nickel may coordinate to RNase through backbone and side chain interactions (*5*) although the proposed formation of Ni(III) would require stabilization afforded by the peptide-like interactions of a deprotonated amide backbone (*8*). RNase has previously been shown to bind Cu^{2+} and Zn^{2+} at multiple sites, but simultaneous ligation expected from both active site histidines (H12 and H119) was not detected (*64, 65*). Interactions with Cu^{2+} and Mn^{2+} have since been characterized by ^{13}C-NMR studies of RNase (*66, 67*). These again indicate variable association with H12, H119, and also H105 and no direct ligation to tyrosine residues. Possible coordination to the polypeptide backbone was not examined then, and visual inspec-

[2] Cysteine thiolates are expected to be additional targets of oxidation, but their disulfide derivatives would be much less reactive. RNase contains six cysteine residues that form three disulfide bonds. Free cysteine was not examined in the competition studies since quenching could result from its avid binding to nickel, its facile oxidation, or both.

tion of the RNase A crystal structure does not reveal any obvious sites for this other than the N-terminus. As an alternative, nickel might coordinate to an accessible and disordered region of the RNase backbone, but no such regions have been identified (*47, 68*−*70*).

RNase A contains six tyrosine residues of which four are accessible to chemical modification at pH 10 (*68*). Y73, Y76, and Y115 cluster at one surface and Y92 resides on a distant surface. Any of these may participate in dimerization, but for steric considerations, further oligomerization to trimers, tetramers, etc. would likely involve Y92 and only one of the three neighboring residues Y73, Y76, and Y115. Tyrosine 73 seems least likely of the three to undergo intermolecular reaction since it appears least exposed on the protein surface. However, the phenolic ring of this residue is directly adjacent to that of Y115, and intramolecular reaction might have been expected. Amino acid analysis indicates that this process was disfavored, and little dityrosine was detected in the monomeric protein recovered after oxidation (Table 1).

Conclusion

Although simple nickel salts are not often associated with oxidation under aqueous conditions, their peptide complexes can be quite reactive with a range of oxidants. Such complexes are thought to facilitate the cellular distribution of nickel as well as promote protein and nucleic acid degradation that can induce allergic, toxic and carcinogenic responses. RNase A served as a model for examining the ability of Ni^{2+} to promote localized and selective oxidation of a protein which, in this example, resulted in polypeptide oligomerization through tyrosine coupling. Radical trapping agents provided little protection against protein cross-linking in the presence of Ni^{2+}, and diffusible Ni(II) macrocyclic complexes used previously for DNA oxidation did not mimic the activity of free nickel. Protein oxidation is likely mediated by transient formation of a Ni(III) intermediate that abstracts an electron from neighboring residues. The ultimate reaction induced by this process may then depend on the available electron donors which in turn contribute to the deleterious effects of nickel exposure.

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