a water bath at 50 °C for 3 min, cooled for 3 min, and finally brought to 0 °C on an ice-bath. Next, a freshly prepared solution of 2-MeImpG (3 μL, 7.1 OD/μL, 0 °C) was added to the tube. The solution was stirred, and the reaction mixture was allowed to stand at 0 °C. After an appropriate time, an aliquot (1 μL) of the solution was transferred to a tube containing 50 μL of water and 1 μL of 0.5 M EDTA (pH 8). Samples (10 μL) of the resulting solution were evaporated to dryness, redissolved in 5 μL of water, mixed with 5 μL of loading buffer, and subjected to electrophoresis on polyacrylamide gel.

Reactions were also carried out at 25, 37, and 50 °C using 2,6-lutidine buffer at pH 8. A few experiments were carried out using essentially the same procedure but which other buffers at various pHs. We also studied the concentration dependence of the reaction in 2,6-lutidine buffer at pH 8 and 0 °C.

Determination of the Half-Times for the Addition of the First Guanosine Nucleotide to Templates I and II. Aliquots (1 μL) of the reaction mixture were withdrawn at appropriate times and transferred to a tube containing 50 μL of water and 1 μL of 0.5 M EDTA (pH 8) and then subjected to electrophoresis on polyacrylamide gel as described above. Bands on the gel were sliced out, and the amounts of radioactivity that they contained were quantitated on a scintillation counter. Half-lives for the first addition of a G residue to the template were estimated by plotting as a function of time the ratio of counts remaining in the band corresponding to starting material to the total counts applied to the gel. No allowance was made for the small amount of hydrolysis of 2-MeImpG that occurs during the earlier stages of the reaction, so the half-lives are approximate.

Isolation of Reaction Products. Reaction products were isolated by ethanol precipitation in the presence of a cold tRNA carrier. The tRNA carrier solution contains tRNA (0.4 mg/mL), ammonium acetate (0.3 M), and EDTA (0.1 mM). It was stored in a freezer at 0 °C.

The reaction mixture (20 μL) was allowed to stand for 24 h at 0 °C, and then 200 μL of tRNA carrier solution and 750 μL of cold ethanol were added. The resulting solution was cooled in acetone/dry ice for 1 h and then centrifuged for 10 min at 0 °C. The supernatant was removed, and the residue (60000 cpm) was dried using a Savant Speed-Vac. The residue was then redissolved in water (5 μL) and loading buffer (5 μL) and separated by electrophoresis on a polyacrylamide gel. The gel was run at 1200 V for about 2 h and visualized by autoradiography on a Kodak film (X-OMAT AR). Bands on the gel corresponding to the major products were sliced out and then soaked in water (200 μL) overnight at room temperature. The supernatant was pipetted off, and the gel was washed with water (2 × 50 μL). These washings were combined with the supernatant and dried on a Savant Speed-Vac. The residue was redissolved in a solution of tRNA mixer (200 μL) and ethanol (95%, 750 μL). The solution was precipitated in a dry ice/acetone bath to give the product (ca. 50000 cpm). The product contains unlabeled yeast tRNA (ca. 1.6 μg of tRNA/1000 cpm).

Alkaline Hydrolysis of Products. A sample of the major product purified as described above (1000 cpm, containing about 1.6 μg unlabeled tRNA) was incubated in sodium hydroxide (5 μL, 0.05 M) on a water bath (55 °C). After 20 min, the reaction was stopped by addition of acetic acid (2.5 μL, 0.1 M). The solution was mixed with the loading buffer (10 μL) and then applied on a polyacrylamide gel.

Ribonuclease T1 Digestion of Products. An aliquot of the purified product (1000 cpm) was dissolved in Tris/EDTA buffer (10 μL, Tris·HCl, 10 mM, pH 7.4; EDTA, 1 mM). The solution was heated in a water bath (90 °C) for 3 min and transferred to a water bath at 55 °C. T1 ribonuclease (1 μL, 100 units) was added, and the solution was incubated for 1 h at 55 °C. The resulting solution was mixed with loading buffer (10 μL) and then analyzed on a polyacrylamide gel.

Ribonuclease H Digestion of Products. The purified product (ca. 10000 cpm) was dissolved in a Tris buffer (20 μL) containing Tris·HCl (0.05 M, pH 8.0), magnesium chloride (0.025 M), potassium chloride (0.1 M), dithiothrietol (1 mM), and sucrose (5% w/v). The solution was incubated at 55 °C for 3 min and then at 32 °C for another 30 min. Ribonuclease H (1 μL, 2 units/μL) was added, and the reaction mixture was left at 32 °C. At appropriate times an aliquot of the solution (4 μL) was withdrawn for analysis by electrophoresis on a polyacrylamide gel.

HPLC Analysis of Products. The reaction was set up as described above but using unlabeled oligonucleotide (1 OD). At appropriate times, an aliquot of the solution (1 μL) was withdrawn and dissolved in 1 mL of water containing 1 mM EDTA. One hundred microliters of this solution was analyzed on an RPC-5 column following a previously described procedure.[16]

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Conformation-Specific Detection of Guanine in DNA: Ends, Mismatches, Bulges, and Loops

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Abstract: DNA oxidation promoted by a square-planar complex of nickel(II) (**1**) in conjunction with $KHSO_5$ provided an excellent method for selectively detecting guanine residues that did not adopt a standard Watson–Crick duplex structure. Sites of modification were indicated by a diagnostic strand scission of DNA induced by subsequent treatment with piperidine. The specificity and, consequently, the utility of this nickel-based reagent were demonstrated through the use of defined oligonucleotide targets. All guanine residues of a random coil reacted readily under the described conditions while the other residues, adenosine, cytidine, and thymidine, remained inert. Most importantly, guanine residues were protected from modification when held within a duplex of complementary paired and stacked bases. This property then allowed for the reliable identification of mispaired, bulged, looped, and terminal guanines from otherwise helical regions of DNA. In addition, the predicted asymmetry of base stacking in a loop structure was confirmed by preferential derivatization of specific guanine residues.

Only a limited set of chemical reactions are currently available for identifying the structural heterogeneity of large DNA and RNA fragments. The physical techniques that have yielded such a wealth of information on oligonucleotide models[1] are rarely applicable to systems of high molecular weight. Accordingly, efforts continue to focus on the design of new reagents for nucleoside modification in order that further details on the variable conformation of polynucleotides may be examined.[2] Our recent

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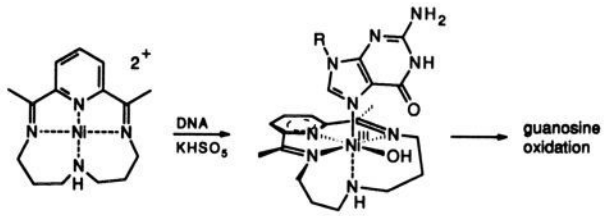
Scheme I



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interest in nickel-dependent reactions of DNA[3] has now led to the discovery of a highly sensitive probe for the structural environment of guanine residues.

Aberrant conformations involving guanine have previously been determined by chemical derivatization of the purine N-1 and 2-amino groups that are left accessible when not involved in hydrogen bonding to cytidine.[2,4] Our approach relies instead on a complementary process, selective oxidation of guanine residues as mediated by the illustrated Ni(II) macrocyclic complex **1** in the presence of $KHSO_5$. This type of reaction has exhibited requirements for coordination and redox chemistry that to date are uniquely provided by nickel.[3] For example, neither platinum nor copper complexes detectably promoted similar transformations of DNA.

A mechanism for the nickel-based oxidation proposed earlier (Scheme I)[3] culminated from published studies on the metal binding properties of DNA[5] and the catalytic abilities of nickel.[6] Steric constraints expected to accompany the suggested ligation between guanine N-7 and nickel in turn predicted an additional level of target selectivity sensitive to DNA structure. In any event, a substantial conformational dependence of DNA modification has now been confirmed by further investigation. The specificity of the nickel complex is described here by its reaction with a series of oligonucleotides that were designed to highlight individual DNA structures and avoid the ambiguities of polymorphic targets.

Results and Discussion

Helical and Nonhelical Guanine Residues. Complex **1** promoted the oxidation of all guanine residues to a similar extent in the random coil formed by the single strand, d-(CATGCGTTCCCGTG).[7] Reaction was detected in each case by diagnostic strand scission induced by subsequent alkaline treatment (lane 3, Figure 1A).[3,8] The resulting DNA fragments appeared to retain phosphate at the 3′-terminus since the fragments co-migrated with the equivalent standards produced by the Maxam–Gilbert method[9] of DNA scission at guanine residues (lane 2, Figure 1A). The net effect of the nickel reagent could be anticipated from two separate reports. First, 8-hydroxy-2′-deoxyguanosine had already been identified as a product of DNA oxidation effected by Ni(II) and H_2O_2,[10] and second, this modified base was independently placed in an oligonucleotide and found to cause strand scission in the presence of alkali and ambient oxygen.[11]

Table I. Sequences and Conformations Used To Characterize the Specificity of the Ni(II)-Dependent Oxidation of DNA[a]

A	5′*CATGCGTTCCCGTG	5′*CATGCGTTCCCGTG 3′ GTACGCAAGGGCAC
B		5′*AGTCTAGTAGACT 3′ TCAGATGATCTGA*
C	5′*ACGTCAGGTGGCACT	5′*ACGTCAGGTGGCACT 3′ TGCAGT-CACCGTGA
D		5′*AGTCTA T G G TCAGAT T G
E		5′*AGTCTC G G TCAGAG G

[a] The underlines and dots indicate sites of maximal and partial reactivity, respectively.

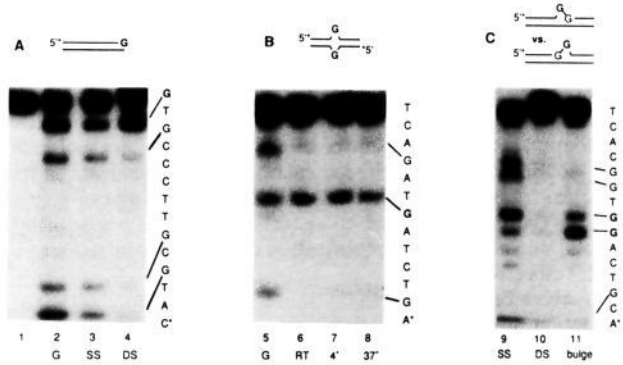

Figure 1. Autoradiograms of denaturing polyacrylamide gels (20%) used to identify the conformation specific oxidation of DNA. Oligonucleotides were labeled with ^{32}P as indicated and, except when noted, were incubated under the standard reaction conditions described in the Experimental Section. (A) Analysis of *d(CATGCGTTCCCGTG): in the absence of **1** (lane 1); Maxam–Gilbert G-lane[9] (lane 2); in the presence of **1** (lane 3) and in the added presence of its fully complementary oligonucleotide (lane 4). (B) Analysis of *d(AGTCTAGTAGACT): Maxam–Gilbert G-lane (lane 5); in presence of **1** at ambient temperature (lane 6), 4 °C (lane 7), and 37 °C (lane 8). (C) Analysis of *d-(ACGTCAGGTGGCACT): in the presence of **1** (lane 9) and in the added presence of its fully complementary oligonucleotide (lane 10) or its deletion derivative d(AGTGCCACTGACGT) (lane 11).

In contrast to the behavior of random-coil structures, duplex DNA was relatively inert to the oxidation promoted by **1**. Only the freely accessible guanine at the 3′-terminus remained a significant target of oxidation when the oligonucleotide above was annealed to its complementary strand (Table I) (lane 4, Figure 1A). Base-stacking and Watson–Crick base-pairing then consistently inhibited productive interaction with the nickel complex.[3] Reaction was only evident for structures that contained either a poorly paired, stacked, or terminal guanosine (see below). In this regard, **1** should now serve as a conformational probe for guanine as diethyl pyrocarbonate has served for adenine.[2,12] The N-7 and C-8 positions of these purines reside in the major groove of standard B-DNA, and yet they are still effectively blocked from reaction. Modification occurs most readily when a structural perturbation significantly enhances the solvent accessibility of these positions.

Guanine–Guanine Mismatch. Reaction of the nickel complex also proved useful in reaction at a guanine–guanine mismatch within a helical region of DNA. Extensive thermodynamic,[13] crystallographic,[14] and magnetic resonance[15] studies indicate that

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guanine residues actually form stable base pairs to guanine, adenine, and thymine as well as to cytidine, the standard Watson–Crick pairing partner. In addition, the unusual base pairs seem to be easily accommodated in duplex DNA and create only minimal distortion of the helical structure. Guanine mismatches have thus been quite difficult to detect in numerous procedures used for medical diagnosis.[16]

Guanine–guanine pairing may now be identified by the hyperreactivity with **1**. This nickel complex induced oxidation of only the single *G* insert in the self-complementary duplex formed by d(AGTCTAGTAGACT) (lane 6, Figure 1B). The internal and correctly paired residues, including the penultimate guanine, were quite inert under these conditions. In contrast, the sterically less demanding reaction, methylation of guanine N-7 by dimethyl sulfate, was unaffected by conformational variation and all sites reacted equally (lane 5, Figure 1B).

The efficiency and selectivity of the Ni(II)-promoted reaction was unchanged in the temperature range 4–37 °C (lanes 6–8, Figures 1B), and therefore control of DNA modification may be traced to a temperature-independent distortion of its duplex structure. If a dynamic phenomenon such as a premelting transition or helical breathing had influenced the reaction instead, then a temperature effect should have been apparent.[17] Finally, the duplex (vs hairpin) nature of the self-complementary sequence was confirmed by the predicted concentration dependence of its thermal transition from helix to coil.[18] The T_m values for this process did indeed increase consistently from 47 to 51 °C using a total oligonucleotide concentration increasing from 1.5 to 11 μM, respectively.

Guanine Bulges. The Ni(II) complex also promoted selective oxidation of unpaired guanine residues in the bulge structures created by hybridization of [5′-^{32}P]-d(ACGTCAGGTGGCACT) and d(AGTGCCACTGACGT) (Table I).[19] In this case, two central guanines were forced to compete for pairing with only a single cytidine. Consequently, both guanines could form a bulge, and both were subject to the oxidative reaction (lane 11, Figure 1C). The appropriate random-coil and fully paired control samples yielded only the predicted profiles; all guanine residues were efficiently modified in the coil and rarely modified in the regular duplex (lanes 9 and 10, Figure 1C).

Also, preferential reaction was observed for one of the two central guanines that could adopt a bulge conformation (lane 11, Figure 1C). This difference may simply reflect the relative equilibrium concentration of the two bulge conformers.[20] No similar distinction can be drawn from the relative exchange rates of the two bulge conformers. Migration of guanine bulges are extremely rapid (≤8 ms)[20] and would only tend to increase the mobility of the participating bases rather equivalently. In this example then, the Ni(II) complex again appears to recognize its targets through access gained by perturbations of DNA structure rather than dynamics.

Guanine Loops. Intrastrand loops containing guanine residues represent another class of structures that is easily characterized

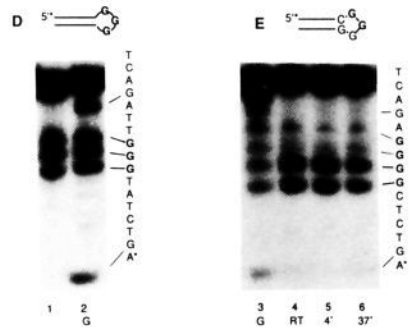

Figure 2. Characterization of DNA hairpin structures by oxidative reaction with **1**. Reaction conditions and product analyses were identical to those described in the Experimental Section. (D) Modification of *d(AGTCTATGGGTTAGACT) by **1** (lane 1) and the corresponding Maxam–Gilbert G-lane (lane 2). (E) Maxam–Gilbert G-lane of *d-(AGTCTCGGGGAGACT) (lane 3) and modification of this sequence by **1** at ambient temperature (lane 4), 4 °C (lane 5), and 37 °C (lane 6).

by the action of **1**. The oligonucleotide d(AGTCTATGGGTTAGACT) spontaneously folded in solution to form a unimolecular hairpin containing five bases in the loop, –TGGGT– (Table I).[19] In this case, all three guanines of the loop were readily oxidized while the two guanines of the stem were completely inert (lane 1, Figure 2D). Since each guanine of the loop reacted to the same extent, no higher order structure was evident for this region. As before, methylation of guanine at N-7 was completely insensitive to base conformation in the stem and loop (lane 2, Figure 2D).

Most importantly, the nickel-dependent oxidation did reveal a higher order of structure in a related oligonucleotide, d-(AGTCTCGGGGAGACT). This sequence was designed with a shorter loop region provided by –GGG– and a stronger loop closure provided by a G–C (vs A–T) pair (Table I).[19] The two guanine residues toward the 5′-end of this central sequence clearly demonstrated the heightened reactivity with **1** that is expected for nonhelical nucleotides (lane 4, Figure 2E). However, the final guanine of the loop was shielded from facile oxidation. Empirical rules now being developed for base-stacking within a loop[21] would indicate accordingly that this base alone could benefit from protection afforded by its stacking between the two neighboring guanines.

The signature modification of this hairpin was also unaffected by temperatures ranging from 4 to 37 °C (lanes 4–6, Figure 2E). This action then remains consistent with the general behavior of **1**. For all examples to date, recognition and derivatization of DNA were stimulated by the absence of standard base-pairing and base-stacking. Temperature-dependent processes were not effective at modulating the oxidation of guanine residues below 37 °C. Chemical modification by **1** should therefore provide a convenient method for identifying the salient characteristics of aberrant nucleotide structure.

Conclusion

The described oligonucleotide studies readily establish the Ni(II) complex **1** as a highly desirable probe for nucleic acid conformation. This reagent selectively promotes a net fragmentation of DNA at guanine residues held accessible through the formation of unusual secondary structures. Hyperreactive sites include random coils, duplex termini, base mismatches, bulges, and loops. Immediate application of this reagent may now be found in the characterization of mutagenic hot spots,[22] telomeric sequences,[23]

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(19) The duplex and hairpin nature of the sequences presented have been confirmed by the respective presence or absence of a DNA concentration dependence on the structures' thermal denaturation (T_m). For the bulge-containing duplex (Table I), values of T_m increased from 48 to 53 °C when the total oligonucleotide concentration was raised from 1.1 to 7.8 μM. In contrast, the T_m values of the single-strand hairpin structures remained relatively constant. The hairpin that contained the –TGGGT– loop exhibited T_m values ranging from 49 to 50 °C over total oligonucleotide concentrations of 1.2–7.1 μM. Similarly, the hairpin containing the –GGG– loop exhibited T_m values ranging from 61 to 62 °C over total oligonucleotide concentrations of 1.2–7.0 μM.

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and biologically active structures of RNA.[24]

Experimental Section

Materials. Oligonucleotides were synthesized via standard solid-phase cyanoethyl phosphoramidite chemistry on DuPont and Biosearch equipment. The desired sequences were then purified to homogeneity under strongly denaturing conditions (pH 12) using anion-exchange chromatography.[25] The Ni(II) complex **1**, [2,12-dimethyl-2,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17),2,11,13,15-pentaene]nickel(II) perchlorate, was synthesized according to published procedures.[26] The terminal oxidant, $KHSO_5$, was obtained from Aldrich. T4 kinase was purchased from BRL, and [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham. All aqueous solutions were made with purified water (Nanopure, Sybron/Barnsted) and reagents of the highest commercial quality.

Preparation and Reaction of DNA Samples. The concentrations of oligonucleotide stock solutions were calculated from their absorbance at 260 nm and the corresponding ϵ_{260} values estimated from the sum of nucleotide absorptivity as affected by the adjacent bases.[27] The indicated sequences (*) were then labeled at their 5'-terminus with ^{32}P using T4 kinase and [γ-^{32}P]ATP. Duplex structures were annealed by combining each oligonucleotide (3 μM) in a solution of 100 mM NaCl and 10 mM potassium phosphate (pH 7) and then placing this mixture in a water bath heated to 90 °C. After 3 min, the bath was turned off and the samples were allowed to cool along with the bath under ambient conditions (>3 h).

Each nickel-based reaction (100 μL) contained 3 μM of a labeled oligonucleotide (10 nCi), 3 μM **1**, 60 μM $KHSO_5$, 100 mM NaCl, and 10 mM potassium phosphate (pH 7). This mixture was maintained under ambient conditions (except when noted) and quenched after 30 min with 20 mM Na_2SO_3. Samples were then individually dialyzed against 1 mM EDTA pH 8 (2 × 3 h) and water (1 × 12 h), lyophilized, treated with 0.2 M piperidine (60 μL) for 30 min at 90 °C, lyophilized again, and resuspended in 80% formamide containing a 0.1% xylene cyanole and bromophenol blue. Maxam–Gilbert G-specific sequencing reactions were performed by routine protocols.[9] Product fragments of DNA were analyzed by 20% polyacrylamide gel electrophoresis under denaturing conditions (7 M urea) and identified by autoradiography using Kodak X-Omat AR5 film.

Thermal Denaturation of Oligonucleotide Secondary Structure (T_m). Optical melting curves were recorded at 260 nm on a Perkin-Elmer lambda 5 spectrophotometer. Sample solutions were related to those used in the modification studies and contained between ca. 1 and 7 μM total oligonucleotide, 100 mM NaCl, and 10 mM potassium phosphate (pH 7). The T_m values were determined as $^1/_2\Delta A_{260}$.

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Registry No. 1, 35270-39-4; $KHSO_5$, 37222-66-5; guanine, 73-40-5.

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Mechanistic Studies on the Inhibition of Thermolysin by a Peptide Hydroxamic Acid

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Abstract: The mechanism of inhibition of thermolysin by the peptide hydroxamic acid HONH-isobutylmalonyl-Ala-GlyNH_2 has been probed by pH and temperature dependencies and solvent deuterium isotope effects. We found the following: (1) At pH 6.5 and 25 °C, the K_i for inhibition of thermolysin by HONH-isobutylmalonyl-Ala-GlyNH_2 is 63 ± 5 nM and reflects a potency for this compound not previously appreciated. (2) The pH dependence of $1/K_i$ at 25 °C is bell-shaped with pK_{a1} = 5.4 ± 0.1, pK_{a2} = 8.2 ± 0.1, and $(K_i)_{limit}$ = 56 ± 4 nM. These pK_a values are similar to those that we obtained from the pH dependence of k_c/K_m for the thermolysin-catalyzed hydrolysis of 3-(2-furyl)acryloyl-Gly-Leu-Ala (hydrolysis at Gly-Leu) and suggest that the active site amino acid residues that are involved in catalysis are also involved in binding this inhibitor. The pH dependence of $1/K_i$ also indicates that thermolysin binds the inhibitor as the neutral, un-ionized acid and not as an anion, as suggested previously by other workers [Holmes, M. A.; Matthews, B. W. *Biochemistry* **1981**, *20*, 6912. Nishino, N.; Powers, J. C. *Biochemistry* **1978**, *17*, 2846. Nishino, N.; Powers, J. C. *Biochemistry* **1979**, *18*, 4340]. (3) At pH 6.5, values of K_i increased with increasing temperature from 18 nM at 5 °C to a plateau of 200 nM between 45 and 60 °C. The van't Hoff plot of this data was analyzed according to a two-step model involving the formation of an initial complex, $(E:I)_1$, that undergoes a conformational isomerization to a second complex, $(E:I)_2$, at high temperature. At temperatures less than 35 °C, only $(E:I)_1$ accumulates and, thus, entirely accounts for inhibition at temperature less than 35 °C. (4) The solvent deuterium isotope effect on K_{ass} ($=K_{ass,H_2O}/K_{ass,D_2O}$, where $K_{ass} = 1/K_i$) is 0.74 ± 0.02 and, like solvent isotope effects for TLN catalysis [$^{D_2O}(k_c/K_m)$ = 0.74; Izquierdo, M.; Stein, R. L. *J. Am. Chem. Soc.* **1990**, *112*, 6054], originates from the transfer of a zinc-bound water molecule to bulk solvent. Based on these results, a mechanism for the inhibition of thermolysin is formulated and discussed.

Introduction

Peptide-derived hydroxamic acids are a class of metalloproteinase inhibitors that are of both medicinal interest, due to the possible involvement of these enzymes in human disease,[1-3] and mechanistic interest, due to insights that might be gained from studying stable complexes of enzymes and these inhibitors.[4-6] The interaction of one of these inhibitors, HONH-benzylmalonyl-L-

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