genation in which the osmium-olefin stereochemistry is conserved.[14] Presumably, hydrogenation occurs exo to the metal center (Figure 1). The observance of residual spin density at 2.68 and 1.52 ppm could be a result of either proton scrambling or interconversion of the metal-olefin bond.

Although an acetonitrile solution of **3** fails to surrender a significant amount of cyclohexene over a 36-h period,[15] its oxidation by $[Fe(Cp)_2]^+$ results in the rapid formation of $[Os(NH_3)_5(CH_3CN)]^{3+}$. The liberation of the olefin is experimentally confirmed by repeating this oxidation in acetone-d_6.[16] Over an 18-h period, an *N*,*N*-dimethylacetamide (DMA) solution of the complex $[Os(NH_3)_5(\eta^2\text{-}C_6H_{10})](OTf)_3$, generated from **3** and AgOTf, quantitatively converts to the solvent complex[17] which is then reduced in the presence of benzene to regenerate **1**. Thus a cycle is completed (Figure 2), in which benzene is hydrogenated to cyclohexene with negligible loss of the pentaammineosmium metal center.

The benzene ligand in the complex $[\{Os(NH_3)_5\}_2(\eta^2{:}\eta^2\text{-}\mu\text{-}C_6H_6)]^{4+}$ has a single olefinic site which is not ligated to osmium.[3] However, attempts to hydrogenate **2** under the reported conditions failed to produce detectable amounts of any reduction product. The catalyst recovered from this reaction was shown still to be active toward hydrogenation of **1**. The expected compound $[\{Os(NH_3)_5\}_2(\eta^2{:}\eta^2\text{-}\mu\text{-}C_6H_8)](OTf)_4$ can readily be generated by conventional methods[18] and is stable in solution for hours. The inert nature of **2** toward hydrogenation under the present conditions is attributed to steric crowding from the metal centers which prevents effective π overlap between the organic ligand and the metal surface (Figure 1).

Although the metal center is conserved in the cycle shown in Figure 2, the process is not catalytic: due to the inert nature of **3**, the removal of cyclohexene is conservatively achieved[19] only through oxidation of the metal. However, the cogener to **3**, $[Ru(NH_3)_5(\eta^2\text{-}C_6H_{10})](OTf)_2$, can be prepared[20] and readily surrenders its organic ligand in MeOH ($t_{1/2} \approx 15$ min at 20 °C). Seeking a catalytic route to cyclohexene, we attempted to hydrogenate benzene in a solution of pentaammineruthenium(II). Though the ruthenium analogue to **1** is not known, we hoped to intercept a metastable η^2-coordinated benzene complex. Several reactions were performed in which $Ru(NH_3)_5(OTf)_3$, Pd/C, and excess benzene were slurried in CD_3OD under H_2.[21] ^1H NMR, CV, and GCMS analysis of the resulting filtrates showed complete conversion of benzene to cyclohexane and formation of significant amounts of ammonium ion;[22] thus far our attempts to isolate the proposed intermediate $[Ru(NH_3)_5(\eta^2\text{-}C_6H_{10})]^{2+}$ or any other pentaammineruthenium salt have been unsuccessful.[23]

Our investigation of monosubstituted benzene derivatives of **1**[3b] indicates that pentaammineosmium(II) displays a high regioselectivity in many of these complexes with activation barriers to tautomerization as high as 17 kcal/mol. It may therefore be possible to achieve stereoselective partial hydrogenation of substituted arenes, in which the facilitating metal center can be conveniently recycled. We hope to extend our investigation to include the selective hydrogenation of other pentaammineosmium(II) complexes of η^2-bound aromatic ligands such as pyridines,[5f] pyrroles, and furans.[24]

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(14) ^1H NMR of **3**-d_4 (acetone-d_6, ppm vs TMS) 3.40 (m, 2 H), 2.68 (m, 0.2 H), 1.52 (m, 0.2 H), 1.40 (m, 1.8 H), 1.12 (m, 1.8 H), 2.88 (b, 12 H), 3.95 (b, 3 H). Exclusive cis hydrogenation of **1** would conserve the mirror plane in the product (**3**-d_4), and the corresponding NMR would feature only three cyclohexene resonances, provided that the metal center was confined to a single side of the hexene plane.

(15) After 36 h at 30 °C an acetonitrile solution of **3** was shown by cyclic voltammetry to contain less than 10% of the complex $[Os(NH_3)_5(CH_3CN)]^{2+}$.

(16) An acetone-d_6 solution of **3** is treated with 1 equiv of $[Fe(Cp)_2]PF_6$. A ^1H NMR of the reaction mixture shows the formation of free cyclohexene (5.75, 2.05, 1.71 ppm).

(17) $[Os(NH_3)_5(\eta^2\text{-}C_6H_{10})](OTf)_3$ is generated from the oxidation of **3** by AgOTf in acetone. The solvolysis of this material in DMA is monitored by cyclic voltammetry.

(18) The complex $[\{Os(NH_3)_5\}_2(\eta^2{:}\eta^2\text{-}\mu\text{-}C_6H_8)](OTf)_4$ was prepared by using synthetic procedures outlined in the following: Harman, W. D.; Taube, H. *Inorg. Chem.* **1987**, *26*, 2917.

(19) I.e., without the subsequent decomposition of the metal center.

(20) The complex $[Ru(NH_3)_5(\eta^2\text{-}C_6H_{10})](OTf)_2$ was prepared by the reaction of cyclohexene with a methanol solution of $[Ru(NH_3)_5(CH_3OH)]^{2+}$.

(21) In a typical reaction $Ru(NH_3)_5(OTf)_3$ (200 mg) and benzene (1.0 mL) are slurried with 5% Pd^0/C (25 mg) in CD_3OD (4.0 g) under 50 psi of hydrogen gas for 1.5-7 h. Under similar conditions $[Ru(NH_3)_6]^{3+}$ is readily reduced by H_2 to $[Ru(NH_3)_6]^{2+}$; the triflate analogue is expected to behave similarly.

(22) A significant amount of partially deuteriated cyclohexanes were also detected.

(23) Efforts are currently directed toward identifying the active form of this ruthenium catalyst.

(24) These complexes have been prepared by using the synthetic procedures outlined in ref 18 and will be reported separately.

Transition-State Structural Features for the Thermolysin-Catalyzed Hydrolysis of *N*-(3-[2-Furyl]acryloyl)-Gly-LeuNH$_2$

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Thermolysin is a metalloendoproteinase isolated from *Bacillus thermoproteolyticus*.[1] Despite extensive kinetic,[2-6] crystallographic,[7-10] and molecular modelling[11] studies, several important mechanistic problems remain unsolved. Resolution of these issues are essential to an accurate description of catalysis by thermolysin and related metalloproteinases. Of central importance is the identification of the rate-determining step and the structural characterization of the rate-limiting transition state.

In this communication, I report results of experiments that probe structural features of the rate-limiting transition state for the thermolysin-catalyzed hydrolysis of the chromogenic substrate, *N*-(3-[2-furyl]acryloyl)-Gly-LeuNH$_2$[12] (FA-Gly-LeuNH$_2$; cleavage occurs at the Gly-Leu bond). Specifically, β-deuterium isotope effects (β-DIE; ratio of rate constants for the hydrolysis of FA-Gly-LeuNH$_2$ and FA-Gly(d_2)-LeuNH$_2$) and solvent deuterium isotope isotope effects (SIE; ratio of rate constants for the hydrolysis of FA-Gly-LeuNH$_2$ in light and heavy water) were determined for the kinetic parameter, k_{cat}/K_m (k_E). Together, these isotope effects suggest that the rate-limiting step is decomposition of a zwitterionic tetrahedral intermediate.

Thermolysin, FA-Gly-LeuNH$_2$, and D_2O were from Sigma Chemical Co. and used without further purification. The deuteriated derivative of the substrate, FA-Gly(d_2)-LeuNH$_2$, was prepared by Bachem. The material appeared isotopically and chemically pure as judged by fast-atom bombardment mass spectroscopy and thin-layer chromatography, respectively. "Kinetic purity"[13] of substrates was verified by the lack of a

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Table I. Kinetic Isotope Effects for the Thermolysin-Catalyzed Hydrolysis of N-(3-[2-Furyl]acryloyl)-Gly-LeuNH$_2$[a]

$[S]_0$ (10^{-6} M)	$^{H}k_E/^{\beta D}k_E$	$^{H_2O}k_E/^{D_2O}k_E$
50	0.956 ± 0.017	0.78 ± 0.02
80	0.967 ± 0.018	0.81 ± 0.01
100	0.960 ± 0.021	0.79 ± 0.03
	0.961 ± 0.016	0.80 ± 0.02

[a] Kinetic experiments were conducted at 24.8 ± 0.1 °C and in a pH 7.48 buffer containing 0.10 M HEPES, 0.01 M $CaCl_2$, and 1.7% DMSO. The enzyme was prepared as a 3 mg/mL stock solution in 0.10 M HEPES, 0.01 M $CaCl_2$, and 2.5 M NaBr and buffered at pH 7.5. Final enzyme concentration in reaction solutions was approximately 0.2 μM. Reaction progress curves for the hydrolysis of the Gly-Leu bond were monitored at 322 nm ($\Delta\epsilon_{322}$ = −2300). Since in these experiments $[S]_0 \ll K_m > 10$ mM, the reactions were first-order in substrate and could be fit, by nonlinear least squares, to a simple exponential rate law. β-DIE[15,16] and SIE[20] were determined as detailed in previous publications.

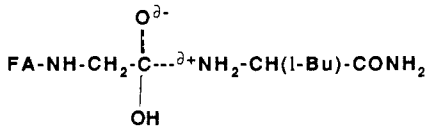
dependence of (i) k_E on the concentration of FA-Gly-LeuNH$_2$ (k_E = 20 100 ± 700 M^{-1} s^{-1}, 30 < $[S]_0$ < 150 mM); (ii) k_E on the concentration of FA-Gly(d_2)-LeuNH$_2$ (k_E = 21 000 ± 500 M^{-1} s^{-1}, 30 < $[S]_0$ < 150 uM); and, (iii) the β-DIE on substrate concentration (see Table I).

The β-DIE for k_E is 0.961 ± 0.016 (Table I) and reflects the loss of hyperconjugation that occurs as the sp^2-hybridization of the substrate in its ground state changes to the partial sp^3-hybridization of the substrate in the tetrahedral intermediate-like transition state.[14,15] The transition state for the thermolysin-catalyzed hydrolysis of FA-Gly-LeuNH$_2$ must therefore resemble the tetrahedral intermediate that occurs along the reaction pathway during amide bond hydrolysis.

The SIE for k_E is 0.80 ± 0.02 (Table I) and stands in marked contrast to solvent isotope effects for serine protease-catalyzed reactions, which typically range from 2.5–3.5.[16] These latter effects reflect the general acid/general base catalysis that promotes acyl transfer to and from the active site serine. The isotopic silence observed here for thermolysin suggests an absence of protolytic catalysis in the transition state for k_E.[17]

We see then, that the rate-limiting step for the thermolysin-catalyzed hydrolysis of FA-Gly-LeuNH$_2$ must involve heavy atom rearrangement ($^{H}k_E/^{\beta D}k_E$ = 0.96) unaccompanied by proton transfer ($^{H_2O}k_E/^{D_2O}k_E \sim 1$). In the context of the current view of thermolysin catalysis,[2-6,11] this combination of isotope effects allows several potential rate-limiting steps to be excluded immediately: (i) formation of the initial encounter complex; (ii) conformational isomerization of this complex; (iii) general-base catalyzed attack of water on the substrate to form a tetrahedral intermediate; (iv) general-acid catalyzed expulsion of the amine leaving group during decomposition of the tetrahedral intermediate; and (v) product release. Steps (i), (ii), and (v) need not be considered as potential rate-limiting steps since these processes do not involve heavy atom rearrangement and would not be expected to generate a β-DIE. Likewise, steps (iii) and (iv) cannot

Chart I



be rate-determining since both of these processes are subject to protolytic catalysis and would generate significant normal SIE.

The reaction step that is most consistent with the experimenal results of this study is collapse of a zwitterionic tetrahedral intermediate to yield FA-NH-CH$_2$-COOH and H$_2$N-CH(*i*-Bu)-CONH$_2$. In the transition state for this step (see Chart I), the α-carbon of the scissle bond has sp^3-character since it resembles, to some degree, the tetrahedral intermediate that preceeds it on the reaction path. The sp^3-character of this transition state reduces the hyperconjugation of the glycine β-hydrogens relative to the reactant state of substrate free in solution. This situation generates the observed β-DIE of 0.96. Also in this transition state, the α-nitrogen of the departing amino amide exists in a protonated, partially cationic state.[18] Since protonation of the α-nitrogen is complete before the substrate and enzyme enter into the transition state, this situation is equivalent to specific-acid catalysis. Thus, the product of transition state fractionation factors can be taken as unity, and we can propose that the observed SIE of 0.80 corresponds to the ground state fractionation factor of the active site Zn^{2+}–H_2O.[17]

(13) Isotope effects on k_E are sensitive to impurities in either of the labeled reactants if these impurities can act as competitive inhibitors or activators of the enzyme under study. Since such materials are anticipated to be present at levels below HPLC or TLC detection limits, one must take advantage of their kinetic signatures to detect them. To assume kinetic purity, one must demonstrate that the first-order rate constant determined at low substrate concentration is independent of substrate concentration for both the deuteriated and nondeuteriated materials and that the isotope effect is independent of substrate concentration.

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(18) The mechanism advanced in this paper for tetrahedral intermediate decomposition, involving specific but not general acid catalysis of amine expulsion, has been discussed previously for chymotrypsin-catalyzed reactions[20] and is based on general mechanistic considerations of amide hydrolysis.

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Direct Observation of Multiple Environments for the H_δ but Not the H_ϵ Proton of a Histidine Residue in Staphylococcal Nuclease

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The ^{1}H NMR spectral properties of the H_ϵ protons of histidines are frequently used to study the structural properties of proteins. For example, Dobson and Fox recently demonstrated by ^{1}H NMR spectroscopy of the well-resolved H_ϵ (or C2H) protons of the four histidine residues in Staphylococcal nuclease (SNase) that SNase can exist as an equilibrium mixture of two folded and two unfolded conformations; this conformational heterogeneity was attributed to cis–trans isomerism of Pro 117.[1,2] We have deuteriated the aromatic rings of the phenylalanine, tyrosine, and tryptophan residues of SNase such that the obscured H_δ (or C4H) protons of the four histidine residues can also be directly observed by ^{1}H

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