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Sequence-Specific Ni(II)-Dependent Peptide Bond Hydrolysis for Protein Engineering. Combinatorial Library Determination of Optimal Sequences

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Abstract: Previously we demonstrated for several examples that peptides having a general internal sequence R_N-Yaa-Ser/Thr-Xaa-His-Zaa-R_C (Yaa = Glu or Ala, Xaa = Ala or His, Zaa = Lys, R_N and R_C = any N- and C-terminal amino acid sequence) were hydrolyzed specifically at the Yaa-Ser/Thr peptide bond in the presence of Ni(II) ions at alkaline pH (Krężel, A.; Mylonas, M.; Kopera, E.; Bal, E. *Acta Biochim. Polon.* **2006**, *53*, 721−727 and references therein). Hereby we report the synthesis of a combinatorial library of CH_3CO-Gly-Ala-(Ser/Thr)-Xaa-His-Zaa-Lys-Phe-Leu-NH_2 peptides, where Xaa residues included 17 common α-amino acids (except Asp, Glu, and Cys) and Zaa residues included 19 common α-amino acids (except Cys). The Ni(II)-dependent hydrolysis at 37 and 45 °C of batches of combinatorial peptide mixtures randomized at Zaa was monitored by MALDI-TOF mass spectrometry. The correctness of library-based predictions was confirmed by accurate measurements of hydrolysis rates of seven selected peptides using HPLC. The hydrolysis was strictly limited to the Ala-Ser/Thr bond in all library and individual peptide experiments. The effects of individual residues on hydrolysis rates were quantified and correlated with physical properties of their side chains according to a model of independent contributions of Xaa and Zaa residues. The principal component analysis calculations demonstrated partial molar side chain volume and the free energy of amino acid vaporization for both Xaa and Zaa residues and the amine pK_a for Zaa residues to be the most significant empirical parameters influencing the hydrolysis rate. Therefore, efficient hydrolysis required bulky and hydrophobic residues at both variable positions Xaa and Zaa, which contributed independently to the hydrolysis rate. This relationship between the peptide sequence and the hydrolysis rate provides a basis for further research, aimed at the elucidation of the reaction mechanism and biotechnological applications of Ni(II)-dependent peptide bond hydrolysis.

Introduction

The sequence-specific cleavage of the peptide bond is a crucial procedure in protein engineering and purification. The extreme stability of this bond, with half-life for spontaneous hydrolysis estimated as 350−600 years at neutral pH and room temperature, limits the range of appropriate cleavage reagents.[1] Those used currently include natural or engineered proteolytic enzymes,[2] self-cleaving intein sequences,[3] and chemical agents, such as cyanogen bromide.[4] All these methodologies have specific disadvantages, resulting from side reactions, insufficient sequence specificities, limitations due to specific reaction conditions, or a requirement for tedious procedures of protease removal.

The search for alternative agents for sequence-specific cleavage of peptides and proteins among metal ions and their complexes included redox-active metal ions. They can cleave peptide bonds, especially in the presence of reactive oxygen species (ROS) precursors, e.g., H_2O_2/ascorbate.[5−7] The redox cleavage, while rapid, is area- rather than site-specific and therefore is not suitable for applications requiring homogeneous protein products.

Hydrolytic chemistry of peptide bond has been used with more success in terms of sequence specificity. Many metal ions and metal complexes were found to promote or catalyze the

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hydrolysis of peptide bonds in short peptides.[8−10] In particular, many metal ions promote the hydrolysis of Xaa-Ser peptide sequences (Xaa = any amino acid) by polarizing the peptide carbonyl group by coordination and assisting in its migration to the intramolecular hydroxyl group.[11,12] These reactions, however, have not been demonstrated for downstream peptide bonds in longer peptides or proteins. In contrast, the Cu(II) ions were reported to specifically cleave the Lys_{226}-Thr_{227} peptide bond in the hinge region of human IgG_1.[13] In follow-up studies, the specific Cu(II)-related hydrolysis was demonstrated for peptide bonds preceding Ser-His and Thr-His sequences at elevated temperatures an pH values.[14−16] These studies indicated that the sequence specificity for metal ion-promoted peptide bond hydrolysis can be based on more than one amino acid residue.

Coordinatively unsaturated Pd(II) complexes, such as *cis*-$[Pd(en)(H_2O)_2]^{2+}$, can exchange water for a sulfur atom present in the side chain of Met or Cys or for a His imidazole nitrogen atom. The cleavage of the second peptide bond upstream results from these interactions.[17,18] Pt(II) aqua ion and its complexes, such as *cis*-$[Pt(en)(H_2O)_2]^{2+}$, bind to Cys and Met sulfurs, causing the cleavage of the first peptide bond downstream.[19,20] These reactions proceed at low pH, around 2. Their sequential specificity is limited to single Cys/Met/His residues. Interestingly, cisplatin (*cis*-$[Pt(NH_3)_2Cl_2]$) was found to share the reactivity with Pd(II) complexes, rather than Pt(II) complexes mentioned above, but in a wider pH range.[21] The Trp nitrogen atom was also found to provide an anchor for hydrolytic Pd(II) and Pt(II) complexes.[22,23] The cleavage of peptide bonds directly preceding Ser/Thr-His/Met sequences in human serum albumin (HSA) was accomplished with the use of a Pd(II) complex, in line with peptide studies.[24]

The hydrolytic cleavage of proteins was achieved only in a few other cases. The scission of myoglobin at Gln_{91}-Ser_{92} and Ala_{94}-Thr_{95} peptide bonds was found for a series of Cu(II) compounds.[25] In two separate studies Cu(II) complexes were reacted with bovine serum albumin (BSA), yielding protein fragmentation, rather than specific cleavage sites, in both cases.[26,27] Altogether, the reactions of metal ions presented above share a disadvantage of low sequence specificity, based on one or two adjacent amino acid residues. Therefore, they are suited better for protein fragmentation, e.g., for mass spectrometry, than for selective cleavage of dedicated sequences for protein engineering.

We found that the CH_3CO-Thr-Glu-Ser-His-His-Lys-NH_2 hexapeptide underwent a slow hydrolysis in the presence of Ni(II) ions in a phosphate buffer, at pH 7.4 and 37 °C.[28] A Ni(II) complex of the C-terminal tetrapeptide amide Ser-His-His-Lys-NH_2 was found to be the product of this reaction, with a yield between 3% and 9% after 140 h of incubation, depending on the concentration of Ni(II) ions. The cleavage occurred solely between the Glu and Ser residues. Subsequent studies revealed that a 34-residue peptide and histone H2A, both comprising the above sequence, were hydrolyzed with an identical sequence specificity by Ni(II) ions under analogous conditions, but ca. 7 times faster.[29] Cu(II) ions hydrolyzed this 34-residue peptide with the same specificity as Ni(II) ions, but 3 times slower, while Co(II) and Zn(II) ions were inactive. In further studies on Ala-substituted analogues of CH_3CO-Thr-Glu-Ser-His-His-Lys-NH_2, we found that the Ser residue and the C-terminal His residue were necessary for hydrolysis to occur.[30] Subsequently, we showed that substitution of the Ser residue with a Thr residue maintained the reactivity toward Ni(II) ions.[31] We demonstrated that the reaction proceeded above pH 7, with an acceleration at pH 9−10. Relatively large differences of hydrolysis rates were found for individual sequences studied.

In brief, our prior studies demonstrated that peptides of a general sequence Yaa-(Ser/Thr)-Xaa-His-Zaa, where Yaa = Glu or Ala, Xaa = Ala or His, and Zaa = Lys, could be hydrolyzed specifically at the Yaa-(Ser/Thr) peptide bond in the presence of Ni(II) ions at alkaline pH. On this basis, we hypothesized that some amino acid substitutions in positions Xaa and Zaa could accelerate hydrolysis further, thus providing a specific hydrolysis site comprising a tri- or even tetrapeptide sequence. The addition of Ni(II) ions to such a site would yield an artificial endopeptidase system with a specificity comparable to those of enzymes used in biotechnological practice. We therefore decided to systematically review the effects of substitutions in positions Xaa and Zaa on the rate of hydrolysis of the Yaa-Ser/Thr bond in the Yaa-(Ser/Thr)-Xaa-His-Zaa-sequence. For this purpose, we synthesized a combinatorial library of R_1-(Ser/Thr)-Xaa-His-Zaa-R_2 peptides (R_1 = CH_3CO-Gly-Ala, R_2 = Lys-Phe-Leu-NH_2), studied their Ni(II)-related hydrolysis using MALDI-TOF mass spectrometry, and performed a thorough statistical analysis of relations between the reaction rate and the peptide sequence. We verified the library screening results for selected R_1-Ser/Thr-Xaa-His-Zaa-R_2 peptides using HPLC. The results

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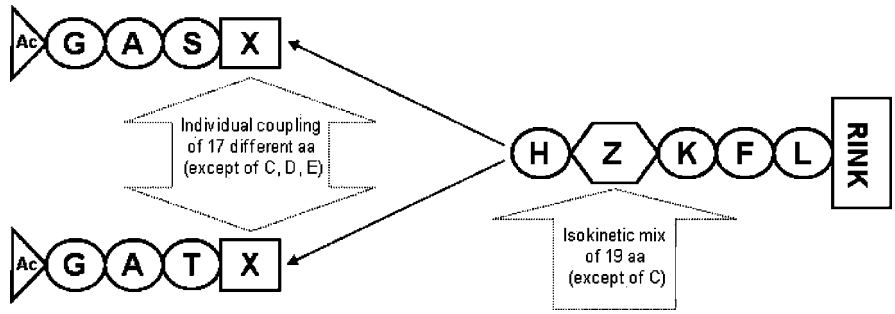
Scheme 1. Design of Synthesis of Peptide Libraries



are described below. In a forthcoming paper (*J. Am. Chem. Soc.*, submitted), we will present a detailed analysis of reaction kinetics and mechanism for these peptides, together with the optimization of reaction conditions for its practical applications.

Experimental Section

Synthesis of Peptide Libraries. The synthesis of the CH_3CO-Gly-Ala-Ser/Thr-Xaa-His-Zaa-Lys-Phe-Leu-NH_2 library was accomplished on an AM-RINK amide resin (NovaBiochem, Merck) on a 800 mg scale, according to a standard manual Fmoc protocol.[32] Residues in position Xaa included the canonical set of standard DNA-coded amino acids, except for Asp, Glu, and Cys, and those in position Zaa included the standard amino acids except for Cys. The peptides were assembled on the resin, starting with the Fmoc-Leu-OH. The Fmoc group was used for temporary protection of the amino group and it was removed before each coupling step with 20% (v/v) piperidine solution in *N*′,*N*′-dimethylformamide (DMF). 1-Hydroxybenzotriazole (HOBt) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (pyBOP) were used as coupling reagents in the presence of DIEA (*N*,*N*-diisopropylethylamine). The resin-attached Leu-Phe-Lys peptide was synthesized first. In the next step, the isokinetic mixture of 19 amino acids selected for position Zaa was coupled,[33] followed by the His residue. The resin carrying the library of pentapeptides was divided into Ser and Thr halves at this stage. Each of these halves was subdivided into 17 portions, which were subsequently coupled with individual amino acids selected for position Xaa. The coupling with Fmoc-Ser(*t*Bu)-OH or Fmoc-Thr(*t*Bu)-OH, as appropriate, and additions of Fmoc-Ala-OH and Fmoc-Gly-OH residues were accomplished subsequently. The acetylation of the N-terminus was performed using acetic anhydrate (Ac_2O) in the presence of DIEA. N-Terminal acetylated resin-attached peptides were cleaved from the resin with the mixture of TFA, AcOH, and DCM 1:2:7 (v/v) followed by evaporation. The library design is presented in Scheme 1.

Synthesis of Selected Peptides. The peptides were synthesized in the solid state according to a standard manual Fmoc protocol (usually 0.5 mg per each peptide with the loading capacity of 0.5 mmol/g) with the 2.5-fold excess of amino acids and coupling reagents diluted in DMF/DCM/NMP (1:1:1) and in the presence of (DIEA) using the AM-RINK (RapPolymere) resin. The pair of HOBt and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) were used as coupling reagents. The coupling reactions were performed in the glass vessels with filters. The Fmoc protection group was removed from growing peptide chains with 20% (v/v) piperidine in DMF followed by a thorough wash with DMF (4×), DCM/DMF (4×), and DCM (4×) in order to remove the excess of piperidine. The progress of amino acid couplings was monitored by the *p*-chloranil test. At the end of chain elongation, the N-terminal amine was acetylated with acetic anhydrate. The resulting N-terminally blocked peptides were cleaved from the resin with a mixture of trifluoroacetic acid (TFA) and a cleavage scavenger reagent, such as triisopropylsilane (TIPS), and water (95:2.5:2.5). The resins were then filtered away, and diethyl ether was added next in order to precipitate the peptide. Each precipitated peptide was filtered through a sintered glass funnel and redissolved in water with acetic acid. The peptide solution was then frozen and lyophilized. Peptides were purified by reverse-phase high performance liquid chromatography (HPLC, Breese Waters) on C18 preparative columns (Vydac). The eluting solvent A was 0.1% TFA/water and solvent B was 0.1% TFA/90% acetonitrile/water. The linear gradient from 10% to 40% in 30 min at a flow rate of 2 mL/min, with detection at 220 nm (common to all peptides) and 280 nm (aromatic residues), was applied routinely. The identities and purities of peptides were confirmed using mass spectrometry, on a Q-Tof1 ESI-MS spectrometer (Waters).

Mass Spectrometry Screening of Hydrolysis Rates. Each of 34 sublibraries randomized in position Zaa was divided into two portions for separate reactions at 37 and 45 °C. The hydrolysis reactions were performed in a 10 mM Tris buffer at pH 8.2. The total concentrations of peptide mixtures and Ni(II) ions were 1 and 2 mM, respectively. The detection of reaction substrates and products was accomplished with the use of a MALDI-TOF (Micromass) mass spectrometer. The aliquots of reaction mixtures were withdrawn and measured at measurement times, t_m, of 0, 2, 4, 6, 8, and 24 h of incubation. The progress of reaction was evaluated by visual detection in mass spectra of the appearance of signals corresponding to expected products of hydrolysis, Ser/Thr-Xaa-His-Zaa-Lys-R_2, at given t_m. The formula used for the semiquantitative evaluation of reaction progress had the form of eq 1.

$$Sc = 24/t_m \quad (1)$$

According to eq 1, a given peptide was assigned a score (Sc) of 12, if a peak corresponding to its predicted hydrolysis product was detected at 2 h, and scores of 6, 4, and 3 were assigned for such detection at 4, 6, and 8 h, respectively. A score of 0 was assigned if no trace of the product of hydrolysis was seen after 8 h of incubation. The data collected at 24 h were not used for the analysis, because the crowding of the spectra made assignments problematic. Equation 1 reflects in a simplified form the pseudo-first-order of the hydrolysis reaction, seen previously for the CH_3CO-Thr-Glu-Thr-His-His-Lys-NH_2 peptide.[31] For the most active peptides, for which the formation of Ser/Thr-Xaa-His-Zaa-R_2 products was seen already at 2 h, the progress of reaction was also controlled at longer incubation times. The decrease of signals of substrates was controlled in these cases, as well.

HPLC Measurements of Hydrolysis Rates. The hydrolysis rates were measured individually for seven selected peptides, R_1-SRHW-R_2, R_1-SKHW-R_2, R_1-SAHW-R_2, R_1-SRHA-R_2, R_1-SGHA-R_2, R_1-TRHW-R_2, and R_1-THHW-R_2, at 37 and 45 °C. The samples contained 1 mM peptide and 1 mM Ni(II) in 20 mM Tris buffer, pH 8.2. The 10 μL aliquots were withdrawn at variable incubation intervals, adjusted individually to reaction times. These aliquots

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Table 1. Hydrolysis Scores and Xaa and Zaa Residue Rankings, Obtained by MALDI-TOF Screening of the Library of 1 mM R_1-Ser-Xaa-His-Zaa-Lys-R_2 Peptides Incubated with 2 mM Ni(II) at 45 °C for 8 h in 10 mM Tris Buffer, pH 8.2

Z→ X ↓	E	D	N	Q	S	A	P	H	T	G	V	Y	M	I	L	K	F	W	R	*Sc*
G	0	0	0	0	0	0	0	0	0	0	3	0	0	3	3	3	0	4	3	19
A	0	0	0	0	0	3	0	4	0	0	3	3	0	4	4	6	3	6	6	42
S	3	3	0	0	3	3	0	3	0	3	4	3	3	6	6	6	3	12	6	67
P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T	0	0	0	0	0	0	0	3	0	0	6	3	0	6	6	6	3	6	6	45
N	0	0	0	0	0	0	0	3	0	0	3	3	0	4	4	4	3	6	6	36
V	0	0	0	0	0	0	0	3	0	0	3	3	0	3	3	4	3	4	3	29
Q	0	0	0	0	0	3	0	3	0	0	4	4	3	6	6	6	4	6	6	51
L	0	0	0	0	0	0	0	0	0	0	3	3	0	3	3	4	3	4	4	27
I	0	0	0	0	0	0	0	3	0	0	3	0	0	3	3	6	0	4	3	25
H	0	0	0	0	0	0	0	4	0	0	6	4	0	6	6	12	3	12	6	59
M	0	0	0	0	3	3	0	3	0	0	6	4	3	12	12	6	4	6	6	68
K	3	0	0	0	0	4	0	3	0	0	6	4	4	12	12	12	4	12	6	82
F	3	0	0	0	0	3	0	6	0	0	6	6	3	6	6	6	6	6	6	63
Y	0	0	0	0	0	0	0	4	0	0	4	4	0	6	6	6	4	6	6	46
R	3	0	0	0	4	6	0	4	3	4	12	6	3	12	12	12	4	12	12	109
W	0	0	0	0	0	3	0	3	0	3	12	6	3	12	12	12	4	6	6	82
Sc	12	3	0	0	10	28	0	49	3	10	84	56	22	104	104	111	51	112	91	850

were mixed with 50 μL of 2% TFA at room temperature to quench hydrolysis and stored at 4 °C until the HPLC analysis. The samples were analyzed on a Breeze HPLC system equipped with an analytical C-18 column (ACE, 250 × 4.6 mm). The substrates and products were separated with elution buffers A (0.1% TFA in water) and B (90% acetonitrile, 0.1% TFA in water), using the gradient of 10−40% buffer B in 15 min, at flow rate of 1 mL/min, and detecting at 220 nm. The peaks were identified on a Q-Tof1 ESI-MS instrument. Pseudo-first-order kinetic constants were calculated using Microcal Origin v. 8.0 (OriginLab).

Statistical Calculations. The library kinetic data were categorized according to the Sc values, which roughly describe the rates of peptide bond hydrolysis for individual library members. Initially, the relationship between Xaa and Zaa contributions into the overall hydrolysis rate was tested separately for each of Tables 1−4 of Sc values (Ser and Thr sets, each at 37 and 45 °C). The zero hypothesis claimed independence of contributions of Xaa and Zaa, which would mean an absence of spatial and electronic interactions between these two residues. In order to test this hypothesis, semiempirical ranks were constructed as products of individual contributions of Xaa and Zaa residues. A positive verification of the hypothesis was obtained using a standard approach based on the Pearson's χ^2-test.[34] The natural logarithms of rate constants obtained in this way for all individual peptides within a given library were then used as smoothed estimates of the free energy of reaction barrier. These kinetic data were subjected to a standard principal components analysis (PCA) procedure, performed iteratively.[35] Various physical and chemical parameters determined for amino acid residues were included in the initial trial set of parameters characterizing the contributions of individual residues (see Results for details).[36,37] In order to balance statistical contributions of individual parameters, all these data were normalized in the PCA analysis according to eq 2.

$$\tilde{x}_i = \frac{x_i - \mu(x)}{\sigma(x)} \qquad (2)$$

where $\mu(x)$ and $\sigma^2(x)$ are the mean and variance of parameter x. After each cycle of calculations the parameter contributing the least to two main eigenvectors corresponding to the largest eigenvalues was purged from the calculations. This was performed to identify various sets of X and/or Z residues that differ in their contribution to the estimated values of effective reaction rates. The PCA analysis was continued until the major parameter, the previously estimated reaction rate, was pointed as the less significant one among the parameters used to distinguish between the peptide clusters. Otherwise, the next steps of parameter reduction would yield a simple clustering of individual residues according to their literature properties, which would not pertain to the analysis of reaction rates. Additionally, after each PCA iteration, the scatterplots built with

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Table 2. Hydrolysis Scores and Xaa and Zaa Residue Rankings, Obtained by MALDI-TOF Screening of the Library of 1 mM R_1-Ser-Xaa-His-Zaa-Lys-R_2 Peptides Incubated with 2 mM Ni(II) at 37 °C for 8 h in 10 mM Tris Buffer, pH 8.2

X \ Z	E	D	N	Q	S	A	P	H	T	G	V	Y	M	I	L	K	F	W	R	Sc.
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	6
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	4	3	10
S	0	0	0	0	0	0	0	0	0	0	3	3	3	6	6	4	3	6	6	40
P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	3	0	4	3	18
N	0	0	0	0	0	0	0	0	0	0	3	3	0	4	4	4	3	6	6	33
V	0	0	0	0	0	0	0	3	0	0	0	0	0	3	3	3	0	4	3	19
Q	0	0	0	0	0	0	0	0	0	0	0	3	0	6	6	4	3	6	6	34
L	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	0	3	3	15
I	0	0	0	0	0	0	0	3	0	0	3	0	0	3	3	3	0	3	3	21
H	0	0	0	0	0	3	0	0	0	3	6	3	0	6	6	6	3	6	4	46
M	0	0	0	0	0	0	0	0	0	0	3	3	3	4	4	3	3	4	4	31
K	0	0	0	0	0	0	0	4	0	0	4	4	3	6	6	6	4	12	4	53
F	0	0	0	0	0	0	0	3	0	0	3	3	0	4	4	4	3	4	4	32
Y	0	0	0	0	0	0	0	4	0	0	6	3	0	4	4	4	3	6	4	38
R	0	0	0	0	3	3	0	4	0	3	6	4	3	12	12	6	4	12	4	76
W	0	0	0	0	0	0	0	4	0	0	3	0	0	6	6	4	3	4	3	33
Sc.	0	0	0	0	3	6	0	25	0	6	40	29	12	71	71	60	32	87	63	505

the two main eigenvectors were subjected to a cluster analysis procedure performed with the aid of the *K*-means algorithm.[38]

Molecular Modeling. The structural calculations were performed for the model 4N square-planar Ni(II) complex of the R_1-SRHW-R_2 peptide using X-PLOR.[39,40] Original X-PLOR "allhdg" parametrization was modified to model the coordination environment of the Ni(II) ion. The lengths of Ni(II)−nitrogen bonds were set to 1.85 and 1.90 Å for backbone amide and histidine side-chain nitrogen, respectively. The complex was kept planar using appropriate improper constraints both for Ni(II) and nitrogen atoms: four nitrogen atoms were kept in the sp^2 hybridization in square planar arrangement around the Ni(II).

The initial complex structure was subjected the simulated annealing protocol (SA), consisting of 6 ps high-temperature evolution and 3 ps cooling down to 300 K, followed by 10 000 steps of energy minimization. This was repeated 999 times, and the 30 lowest energy conformers were chosen as a representative sample of the conformational space. The ensemble of conformers was analyzed with the aid of MolMol.[42]

Results

Principles of Library Design and Screening. The peptide libraries designed for this study had a general sequence R_1-(Ser/Thr)-Xaa-His-Zaa-Lys-R_2. Xaa included 17 common αa-amino acids (except Asp, Glu, and Cys), and Zaa included 19 common α-amino acids (except Cys). For the clarity of description we shall use the "Ser library" and "Thr library" terms for the whole sets of R_1-**Ser**-Xaa-His-Zaa-R_2 and R_1-**Thr**-Xaa-His-Zaa-Lys-R_2 peptides, respectively. The peptides were synthesized in 34 batches, each containing a mixture (sublibrary) of 19 peptides randomized at Zaa, with known Ser/Thr and Xaa residues. Peptide sequences R_1 and R_2 were designed so that the masses of substrates and their expected variable C-terminal hydrolysis products, (Ser/Thr)-Xaa-His-Zaa-R_2, were in the range of 700−1200, optimal for the detection by the MALDI-TOF spectrometer as +1 ions. The molecular mass of the lightest expected C-terminal product of the Ser library was 744 Da, and that of the heaviest substrate of the Thr library was 1187 Da. The spread of masses within clusters of substrates and C-terminal products was 129 Da, equal to the difference between the lightest and the heaviest Zaa substituents, Gly and Trp. Therefore, the constant mass separation between the substrates and these products, equal to 170 Da (CH_3CO-Gly-Ala-OH), assured a good separation and, consequently, easy identification of signals of reaction products. The presence of the Lys residue in the invariable R_2 tail of the C-terminal hydrolysis products assured the sufficient positive ionization of all library members.

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Table 3. Hydrolysis Scores and Xaa and Zaa Residue Rankings, Obtained by MALDI-TOF Screening of the Library of 1 mM R_1-Thr-Xaa-His-Zaa-Lys-R_2 Peptides Incubated with 2 mM Ni(II) at 45 °C for 8 h in 10 mM Tris Buffer, pH 8.2

X \ Z	E	D	N	Q	S	A	P	H	T	G	V	Y	M	I	L	K	F	W	R	Sc.
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3
N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	6
V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	6
Q	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	0	4	4	17
L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3	3	9
I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3	3	9
H	0	0	0	0	4	4	0	4	4	4	6	6	4	12	12	12	6	12	6	96
M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3	4	10
K	0	0	0	0	0	3	0	4	0	0	6	6	4	12	12	12	6	12	12	89
F	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3	4	10
Y	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	4	12
R	0	0	0	0	0	3	0	4	0	0	4	6	4	12	12	12	4	12	6	79
W	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	4	0	4	4	21
Sc.	0	0	0	0	4	10	0	12	4	4	16	18	15	42	42	59	16	66	59	367

The omission of Asp and Glu residues at position Xaa was dictated by the fact that they contain carboxylates in their side chains. Such carboxylates may bind the Ni(II) ion in a way which would quench or slow down the hydrolysis reaction.[43] The Cys residue was eliminated because it was likely to provide alternative Ni(II) binding in either of variable positions, resulting in unwanted coordination heterogeneity. Cysteine peptides are also prone to free-radical redox processes in the presence of Ni(II) ions, which might result in oxidative side reactions.[44−46]

The temperatures of 37 and 45 °C and pH of the reaction of 8.2 were chosen on the basis of our previous studies as relatively mild and at the same time potentially selective for highly active sequences.[28−31] In particular, our previous study of the CH_3CO-Thr-Glu-Thr-His-His-Lys-NH_2 peptide indicated that its Ni(II)-dependent hydrolysis was effective at pH values higher than 8.5 and temperatures higher than 50 °C.[31] Therefore, the detection of substantial amounts of reaction products during library screening would prove the presence of peptides much more susceptible to Ni(II)-dependent hydrolysis than those known to date.

Library Screening and Evaluation. Figure 1 shows a MALDI-TOF mass spectra of a representative sublibrary (R_1-SRHZ-R_2) at 0 and 24 h of incubation at 45 °C. Only +1 ions were observed. Tables 1 and 2 present the Sc values (defined in the Experimental Section), obtained for the Ser library at 45 and 37 °C, respectively. Tables 3 and 4 show the respective values for the Thr library. The residues in these tables were provisionally sorted in rows (Xaa, in short X) and columns (Zaa, in short Z) according to the van der Waals volumes of their side chains and octanol/water partition coefficients, respectively. One can see that these parameters provided a good, but not a perfect, ordering of hydrolytic properties.

On the a priori assumption of the additivity of Xaa and Zaa contributions, the Sc values were summed up in lines and columns of Tables 1−4, thereby creating cumulative scores for substituents in these positions, XSc and ZSc, respectively. Individual peptide scores, ISc, were calculated by adding XSc and ZSc of its substituents. The Ser library provided a higher number of reactive peptides than the Thr library, and the hydrolysis was faster at 45 °C than at 37 °C for both cases. The sums of all Sc values within these individual experiments are presented in Table 5 to illustrate these tendencies. Figure 2 presents correlations of XSc and ZSc values between Ser and Thr libraries. The correlation of XSc values was weak, with $R = 0.62$, while that of ZSc values was strong, with $R = 0.93$.

Measurements of Hydrolysis Rates for Individual Peptides. Seven peptides were chosen for detailed measurements of rates of Ni(II)-related hydrolysis and their temperature dependence. This selection included five Ser library peptides: two of them with top-scoring Xaa and Zaa substituents, R_1-SRHW-R_2 and R_1-SKHW-R_2; two with one top-scoring and one bottom-scoring substituent, R_1-SAHW-R_2 and R_1-SRHA-R_2; and one peptide without top-scoring substituents, R_1-SGHA-R_2. This

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Table 4. Hydrolysis Scores and Xaa and Zaa Residue Rankings, Obtained by MALDI-TOF Screening of the Library of 1 mM R_1-Thr-Xaa-His-Zaa-Lys-R_2 Peptides Incubated with 2 mM Ni(II) at 37 °C for 8 h in 10 mM Tris Buffer, pH 8.2

X \ Z	E	D	N	Q	S	A	P	H	T	G	V	Y	M	I	L	K	F	W	R	Sc.
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Q	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	6
L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3
I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3
H	0	0	0	0	0	0	0	3	0	0	0	0	0	3	3	6	0	12	6	33
M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	6
K	0	0	0	0	0	0	0	0	0	0	3	3	3	4	4	3	3	6	4	33
F	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	3	6
Y	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3	4	10
R	0	0	0	0	0	0	0	3	0	0	3	4	3	6	6	6	3	12	4	50
W	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	3	3	10
Sc.	0	0	0	0	0	0	0	6	0	0	6	7	6	13	13	25	6	48	30	160

set was complemented with two top-scoring Thr library peptides, R_1-THHW-R_2 and R_1-TRHW-R_2. The Ni(II)-dependent hydrolysis of these peptides was studied in a detailed, quantitative fashion under the conditions analogous to those used in library screening. HPLC was used to separate and quantitate reaction substrates and products. Examples of chromatograms are

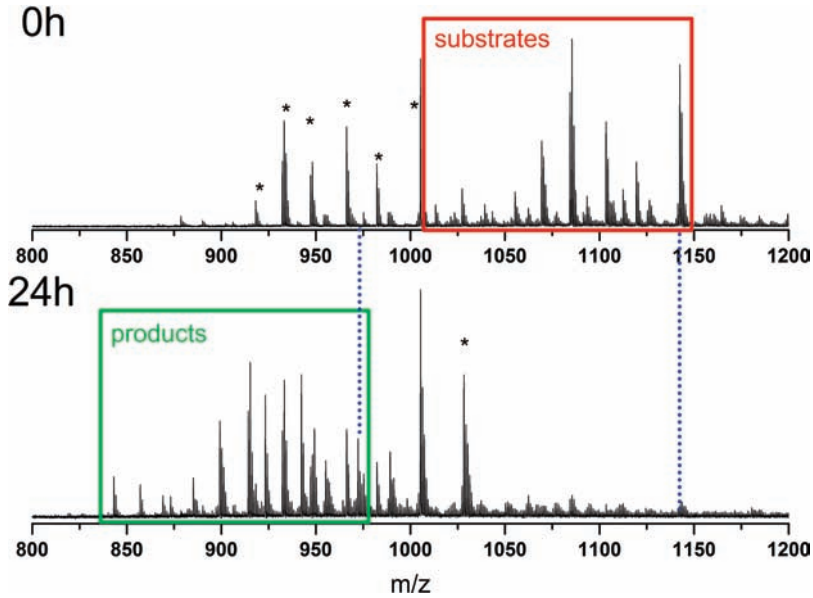

Figure 1. Example of MALDI-TOF spectra of peptide libraries. (Top) MALDI-TOF spectrum of the R_1-SRHZ-R_2 sublibrary, incubated at 45 °C at 0 h (immediately after Ni(II) addition). (Bottom) MALDI-TOF spectrum of the same sublibrary, incubated at 45 °C for 24 h. Red and green boxes mark the spectral regions of substrates and products, respectively. Blue dotted lines mark the positions of R_1-SRHW-R_2 (substrate) and SRHW-R_2 (product) peptides. Asterisks mark major impurities.

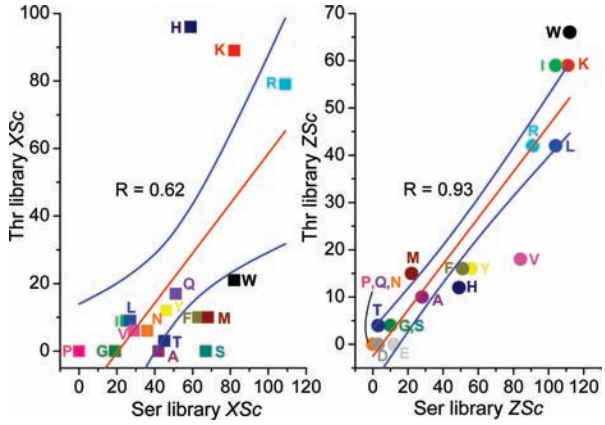

Figure 2. Correlations of cumulative scores at positions X (XSc) and Z (ZSc) between Ser and Thr peptide libraries. Trend lines (red), 95% confidence bands (blue), and linear correlation coefficients *R* are indicated in the plots.

Table 5. Sums of Library Score (Sc) Values for Individual Library Experiments

library	45 °C	37 °C
Ser	850	505
Thr	367	160

presented in Figure 3. Individual peaks were identified by ESI-MS. A substantial amount of a transient reaction product was observed in some, but not all, cases. Its retention time was always between those of the substrate and the final product, while its molecular mass and MS isotopic profile were always identical with those of its substrate. The final reaction product corresponded to the removal of the N-terminal CH_3CO-Gly-Ala-OH dipeptide in all cases, as expected. This dipeptide was not detected in HPLC as a separate peak. Due to its low size it eluted within the dead volume of the column. No peptide bond hydrolysis was observed in control samples, incubated in the absence of Ni(II) ions.

The reaction rates were obtained by fitting the normalized integrals of substrate and C-terminal product peaks to the first-order rate equation. The intermediate products was automatically summed up with their substrates during the MALDI-TOF scoring of peptide libraries, since their masses were identical. That approach was reproduced in these calculations, as illustrated in Figure 4. As mentioned above, the CH_3CO-Gly-Ala-OH product was not observed in HPLC analysis; thus, this hydrolysis product was not included in the calculations. Despite that, the substrate and product integrals were sufficiently similar for all peptides studied. This can be explained by the fact that the CH_3CO-Gly-Ala-OH molecule absorbs light only marginally at the detection wavelength of 220 nm.

The rate constants obtained from the above calculations are presented in Table 6. Figure 5 compares them with ISc values for respective peptides of Ser library. A positive correlation between these two measures of susceptibility to Ni(II)-related hydrolysis is obvious. These constants can also be used to re-estimate Sc values, on an assumption, based on inspection of noise levels in MALDI-TOF spectra, that the threshold of reaction product detection in these spectra corresponded to ca. 20% of product formation. This condition relates $Sc_{estimate}$ values with $t_{1/5}$ of first-order reactions according to eq 3:

$$Sc_{estimate} = 24/t_{1/5}\ [h] \qquad (3)$$

$Sc_{estimate}$ values are also provided in Table 6 to show that correct Sc values were reproduced in most cases. This agreement validated the library scoring methodology and prompted a conclusive statistical analysis of the library data.

Statistical Analysis of Library Scores. The analysis of the categorized set of individual scores determined for the Ser library at 45 °C was performed first, because this experiment provided the highest number of nonzero Sc entries. This analysis was based on an assumption that the contributions of Xaa and Zaa residues are mutually independent. This assumption was justified by the Pearson χ^2-test. This approach enabled us to generate a set of semiexperimental scores. These scores were compared to experimental rate constants. Figure 6 demonstrates an excellent linear correlation between these parameters for all five Ser library peptides. The slope of the correlation, 2.5 ± 0.3, is due to a 2-fold excess of Ni(II) over the mixture of peptides used in library experiments, while only a 20% excess of Ni(II) over the peptide was applied in detailed studies.

Semiempirical scores validated in this way were then subjected to the PCA procedure. Partial molar volumes, molecular volumes, and various polarity coefficients of amino acid residues were included in the initial trial set of parameters characterizing the contributions of individual residues.[36] Stepwise reductions of this initial set in foregoing cycles of PCA analysis yielded models that included partial molar volumes of residues Xaa and Zaa (X_{V2O} and Z_{V2O}, respectively), and free energies of the amino acid side-chain vaporization of residues Xaa and Zaa (X_{vap} and Z_{vap}, respectively).

The peptides were then bifurcated into two groups according to the electronic properties of the Zaa residue. At this stage, the pK_a value of the α-amino group of the free amino acid (Z_{pK_a}) was included as an additional parameter.[37] This parameter significantly improved the prediction of the peptide hydrolytic activity. The detailed analysis of the PCA data of the Ser library proved that the clustering of the peptides was related to the type of Zaa residue. The peptides carrying a Trp, Asp, or Glu residue at Zaa belonged to the minor cluster of highly active peptides, while all other peptides built the major cluster. The peptides from the major cluster were then finally subjected to the generalized linear regression of the PCA-derived parameters against the estimated rate constants. In the Thr library, another subset of highly active peptides could be discerned according to the parametrization obtained for the Ser library. It contained peptides with His, Lys, and Arg residues as Xaa. The separation of the Zaa = Trp set from the major cluster was much smaller (note that Zaa = Asp and Glu yielded null ZSc values for this experiment, and therefore, they were excluded from the analysis). Table 7 presents regression results, which demonstrate a good correlation between the predicted and the semiexperimental values. Minor clusters could not be analyzed in the same way, because their low sizes did not satisfy formal requirements of the linear regression algorithm for the minimal number of independent variables.

Figure 7 shows the regression plots for both Ser and Thr libraries, arranged according to the major clusters (circles). The Z = Trp (and Glu, Asp for the Ser library) peptides are marked with squares, and the X = His, Lys, Arg peptides are marked with triangles. These subsets stay out for the Ser and Thr libraries, respectively, as explained above. Within the major clusters, the largest deviations were observed for the peptides with *Z* = Thr, Ser, but this seems to be an effect of specific interactions of the hydroxyl group, rather than a systematic deviation. The analysis of the parameters selected by PCA

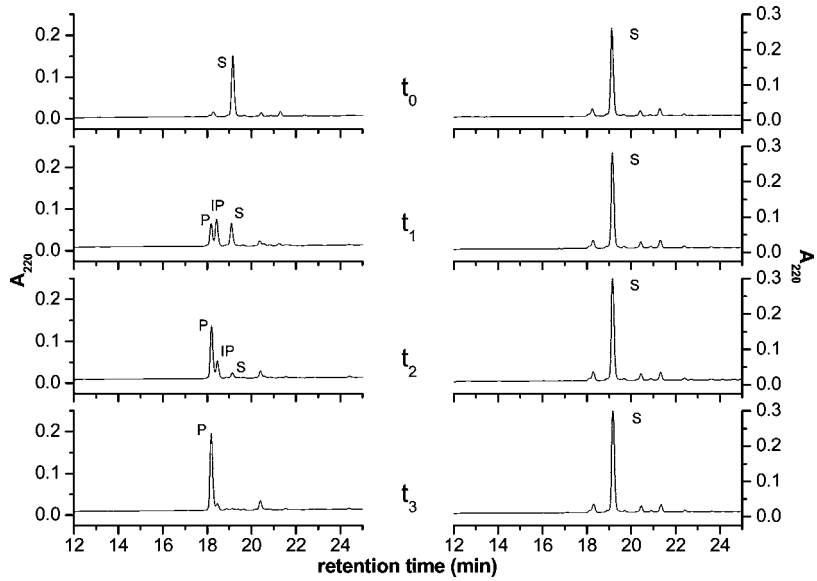

Figure 3. HPLC chromatograms of 1 mM R_1-SRHW-R_2 peptide incubated in 20 mM Tris buffer, pH 8.2 at 45 °C, in the presence (right) or absence (left) of 1.2 mM Ni(II) ions, recorded at incubation times $t_0 = 0$ min (before incubation), $t_1 = 93$ min, $t_2 = 221$, and $t_3 = 384$ min. Peak labels: S, R_1-SRHW-R_2 reaction substrate; IP, intermediate product; P, SRHW-R_2 reaction product.

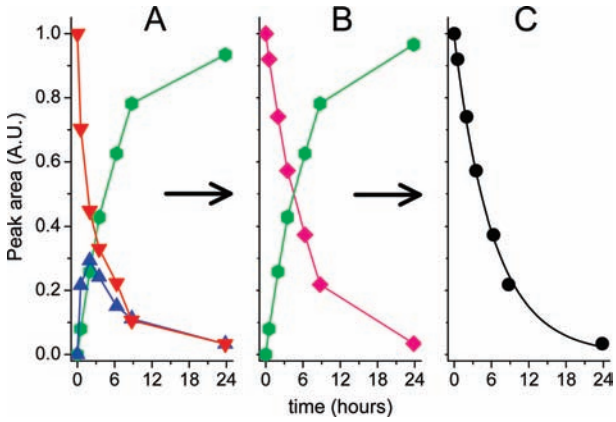

Figure 4. Procedure of calculations of rate constants from HPLC data, presented for the R_1-SKHW-R_2 peptide incubated at 45 °C. (A) relative peak area of the substrate (down triangles), the intermediate product (up triangles), and the SKHW-R_2 reaction product (hexagons). (B) The result of summing up the substrate with the intermediate product (squares). (C) The first-order rate law fit (line) of the summed up data of panel B (circles).

proved that bulky and hydrophobic residues improve the efficiency of hydrolysis at both Xaa and Zaa positions (Table 7). This effect is ca. 3 times stronger for Zaa than for Xaa. Additionally, the high pK_a of the Zaa residue contributed positively to the reaction rate, while the electrostatic effect of the Xaa residue was negligible.

The parametrization optimized for the major cluster of the Thr library was close to that obtained for the Ser library. The only significant difference concerned the effect of the size of the Xaa residue, found to be 3 times stronger than that observed in the Ser library. This is certainly due to a possibility of direct interactions between Xaa side chains and the Thr methyl group. However, looking at the original scores of the Thr sublibrary, one can immediately see that the dominant contribution to the activity of these peptides is confined to the Xaa = His, Lys, Arg subset (cumulative Sc of 264 per total 367 and all Sc = 12 entries). For these three peptides, the Ser major cluster parametrization is appropriate. This is illustrated in Figure 7

Table 6. Comparison of Experimental Rate Constants (k^1) of Hydrolysis of Seven Selected Peptides with Their Library Score (Sc) Values[a]

	37 °C			45 °C		
peptide	k^1 ($s^{-1} \times 10^{-5}$)	Sc	$Sc_{estimate}$	k^1 ($s^{-1} \times 10^{-5}$)	Sc	$Sc_{estimate}$
R_1-SRHW-R_2	4.7(2)	12	18.0 [12]	8.9(6)	12	34.4 [12]
R_1-SKHW-R_2	3.7(3)	12	14.3 [12]	4.5(5)	12	23.2 [12]
R_1-SAHW-R_2	2.0(5)	6	7.6 [6]	2.4(3)	6	9.4 [6]
R_1-SRHA-R_2	1.1(1)	3	4.2 [3−4]	1.5(1)	6	5.9 [4−6]
R_1-SGHA-R_2	0.35(7)	0	1.4 [0]	0.87(8)	0	3.4 [3−0]
R_1-TRHW-R_2	1.5(5)	12	5.7 [4−6]	1.3(3)	12	5.0 [4−6]
R_1-THHW-R_2	2.1(3)	12	7.9 [6]	3.9(8)	12	15.2 [12]

[a] The values in parentheses denote standard errors (SE) on the last significant digit of k^1 values. $Sc_{estimate}$ values were obtained by application of eq 3 to these values. The values in brackets in $Sc_{estimate}$ columns result from the rounding of estimates to the nearest experimentally possible Sc values (12, 6, 4, 3, or 0).

with the trend line. This parametrization overestimates the Thr major cluster peptides' activities approximately 5-fold, thus indicating that the Thr methyl group counteracts the reaction for Xaa ≠ His, Lys, Arg. This parametrization also overestimates the Zaa = Trp (Asp, Glu) contributions in both libraries, by a factor of ca. 3 for the most active peptides of this group, which are located in the top right corner of the plots, and up to nearly 40-fold for the least active ones. Therefore, Trp (Asp, Glu) residues may be involved in cooperative interactions that destabilize the active complex for the majority of Xaa substitutions.

Discussion

Validation of Study Concept. As shown above, certain specific combinations of substitutions in positions Xaa and Zaa of the general sequence R_1-Ser/Thr-Xaa-His-Zaa-R_2 resulted in a dramatic increase of the rate of hydrolysis of the Ala-Ser/Thr peptide bond in the presence of Ni(II) ions, while preserving the sequence specificity of the reaction. Among 646 peptides tested by library screening at 45 °C, only 32 were highly active (Sc = 12), while 413 were inactive (Sc = 0). This proportion was even more striking at more selective 37 °C: 6 highly active

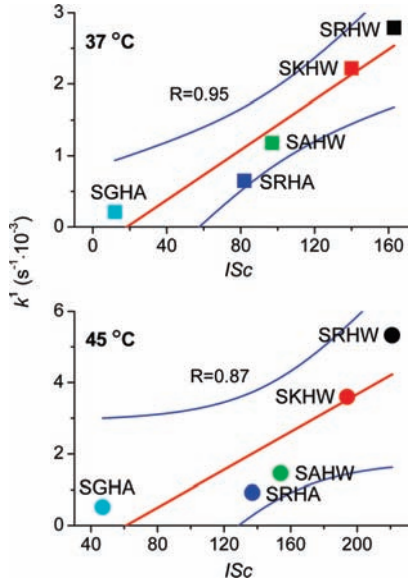

Figure 5. Linear correlations between ISc values and accurate experimental rate constants, determined at 37 and 45 °C for selected R_1-SXHZ-R_2 peptides (labeled using their SXHZ portions). Trend lines (red), 95% confidence bands (blue), and linear correlation coefficients *R* are indicated in the plots.

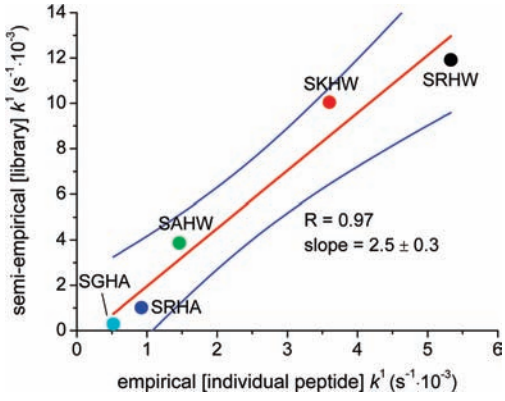

Figure 6. A linear correlation between accurate experimental rate constants and semiempirical library rate constants at 45 °C for selected R_1-SXHZ-R_2 peptides (labeled using their SXHZ portions). Trend lines (red), 95% confidence bands (blue), linear correlation coefficient *R*, and slope of the fitted line are indicated in the plot.

vs 481 inactive (Tables 1−4). Among peptides tested individually, the enhancement of the hydrolysis rate for R_1-SRHW-R_2, compared to R_1-SGHA-R_2, was 10-fold at 45 °C and higher than 13-fold at 37 °C (Table 6). Therefore, the cornerstone hypothesis of the study was tested positively. Moreover, the results of this study contribute significantly to a prospective mechanism of the reaction studied and pave the way for further optimization of reaction conditions.

Structure−Reactivity Relationships. All peptides that underwent hydrolysis in the presence of Ni(II) ions reacted according to the same pattern: only the peptide bond preceding the Ser/Thr residue was hydrolyzed. This fact indicates that residues Xaa and Zaa do not participate directly in the reaction, instead, their participation has a sterical character. Our previous study on the CH_3CO-Thr-Glu-Thr-His-His-Lys-NH_2 peptide[31] indicated that the hydrolysis was enabled by the formation of a characteristic, square-planar complex with the hydrolysis substrate, which contains the Ni(II) ion coordinated to four nitrogen

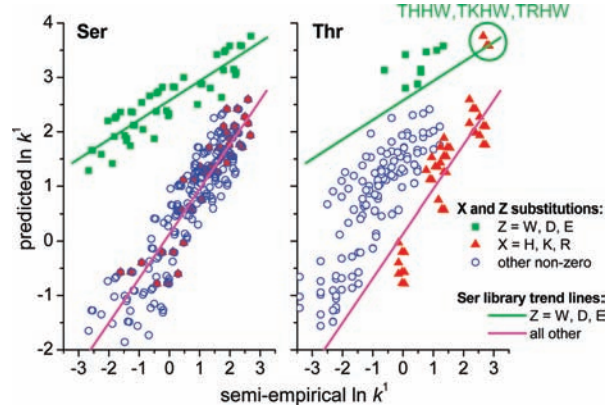

Figure 7. The regression plots of natural logarithms of predicted (Table 7) versus semiempirical reaction rate constants for libraries at 45 °C. Symbols and lines are explained in the graph. Ser library trend lines are projected onto the Thr library to demonstrate special properties of X = H, K, R peptides of the latter. The circle marks the X = H, K, R; Z = W peptides.

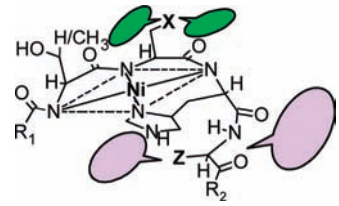

Figure 8. Structural sketch of the proposed hydrolytically reactive $NiH_{-2}L$ complex of a library peptide L. Green and violet clouds indicate areas of influence of Xaa (X) and Zaa (Z) residues, respectively.

donors (4N structure).[47] This structure, presented schematically in Figure 8, is analogous to those well-known for serum albumins and related peptides, including products of Ni(II)-related hydrolysis characterized by us before.[28−31,41,48−51] The only difference is that it includes an amide rather than amine nitrogen, provided by Ser/Thr residue.

The structure in Figure 8 is consistent with the following results of statistical analysis and empirical observations: (1) The 4N structure separates Xaa and Zaa residues spatially, which accounts for the independence and additivity of Xaa and Zaa contributions. (2) The 4N structure cannot be formed with Xaa = Pro.[47−52] In fact, all Sc values were 0 for Xaa = Pro in all library experiments (Tables 1−4). (3) The Xaa side chain in the 4N structure is adjacent to the Ser/Thr side chain.[48] This explains the much higher contribution of Xaa residue to the linear regression over the Thr library, compared with the Ser library (Table 7); the same effect manifested itself in a poor correlation of XSc parameters between Ser and Thr libraries (Figure 2). (4) The side chain of the Zaa residue has a spatial freedom to interact at the bottom of the Ni(II) chelate structure (the top is defined by Ser/Thr and Xaa side chains).[48] This

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Table 7. Linear Regression Parameters for Ser and Thr Libraries at 45 °C[a]

	Ser library Zaa ≠ Trp, Glu, Asp			Thr library Z ≠ Trp[b]; Xaa ≠ His, Lys, Arg[c]		
parameter	value	95% CI limits	*t*-stat	value	95% CI limits	*t*-stat
free	−39(2)	[−43; −36]	−21.7	−31(2)	[−36; −27]	−14.0
Z_{V2O}	0.039(1)	[0.036; 0.042]	27.3	0.035(2)	[0.031; 0.038]	19.5
Z_{pK_a}	3.8(2)	[3.4; 4.2]	20.1	2.7(2)	[2.2; 3.1]	11.4
Z_{vap}	−0.100(10)	[−0.121; −0.080]	−9.6	−0.084(13)	[−0.110; −0.059]	−6.5
X_{V2O}	0.006(1)	[0.004; 0.009]	4.9	0.021(2)	[0.017; 0.025]	10.4
X_{vap}	−0.055(7)	[−0.068; −0.041]	−7.9	−0.035(9)	[−0.053; −0.017]	−3.8

[a] Values in parentheses denote standard errors (SE) on the last significant digit. [b] Glu and Asp were excluded from the analysis because they yielded null cumulative scores. See the text for more details. [c] Hydrolytic properties of these peptides agree with the parametrization estimated for the major cluster in the Ser library.

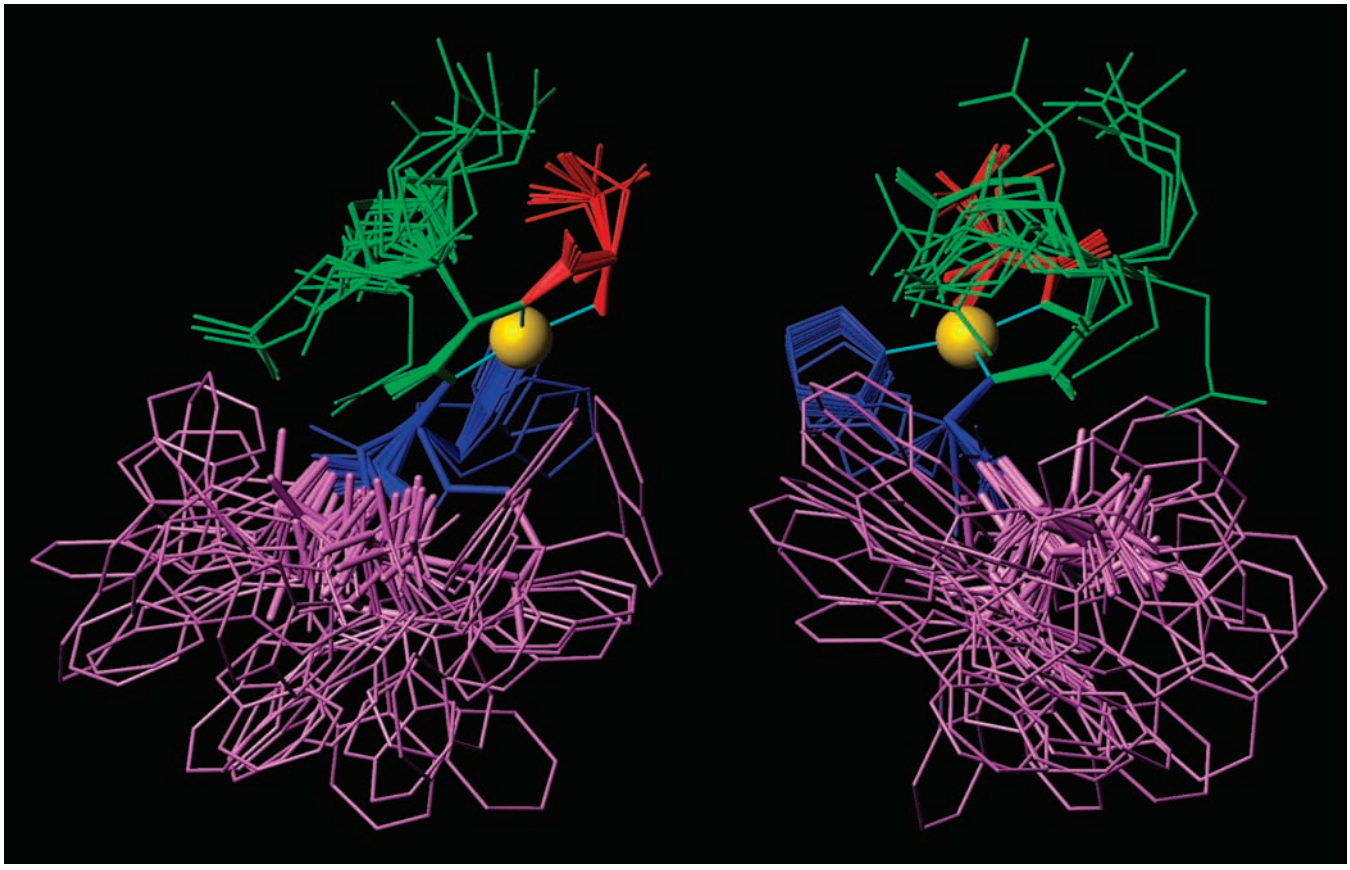

Figure 9. Orthographic projections of the ensemble of 30 lowest-energy structures of the proposed hydrolytically reactive $NiH_{-2}L$ complex of the R_1-SRHW-R_2 peptide. The Ser residue is in red, Arg in green, His in blue, and Trp in violet, while the Ni(II) is in gold and the four N−Ni(II) bonds are in cyan. Other residues are invisible for the sake of clarity.

property agrees well with a very high correlation of ZSc parameters between Ser and Thr libraries (Figure 2). (5) The Pro residue in position Zaa cannot bend back toward the Ni(II) chelate plane due to its rigidity. Accordingly, all Sc values were 0 for this substitution in all library experiments (Tables 1−4).

The above concepts are further corroborated by results of molecular modeling of the 4N ($NiH_{-2}L$) complex of the R_1-SRHW-R_2 peptide, presented in Figure 9. The planar arrangement of nitrogen ligands, enforced by the diamagnetic Ni(II) ion, separates in space the side chains of residues X (here Arg, green) and Z (here Trp, violet). The X residue has a freedom of rotation above the complex plane, while the less restricted Z residue moves freely aside and below the plane.

The PCA and linear regression calculations (Table 7) demonstrated that hydrolysis rates were enhanced for Xaa and Zaa substituents characterized with both high partial molar volumes and low free energies of the side-chain vaporization. These parameters define quantitatively the terms of bulkiness and hydrophobicity, respectively, so the hydrolysis is enhanced by molecular crowding around the Ni(II) ion. Molecular crowding enhances the stability of the 4N complex, according to the mechanism of shielding of Ni(II)−N bonds.[48−53] Therefore, the rate enhancement at pH 8.2 may be at least partially due to the increase of 4N complex stability and therefore its ability to facilitate hydrolysis.

The pK_a of the amino acid is an indirect measure of electronic, rather than sterical properties of the side chain. Trp was found to be the most effective Zaa substitution. The calculations indicated, however, that it underperforms, compared to linear regression predictions, as well as Asp and Glu do. Perhaps, these

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residues can participate in some additional electrostatic interactions that destabilize the active form of the peptide.

Perspectives of Further Studies. Above, we presented the structural concept which rationalizes the relationships between sequences of peptides and their susceptibility to Ni(II) hydrolysis. In a future paper (*J. Am. Chem. Soc.*, submitted), we will verify it by investigating the relationship between the Ni(II) complexation properties of seven selected peptides (Table 6) and their hydrolytic reactivity. We will also research the optimal conditions of the reaction in the perspective of its application for protein engineering.

Conclusions

We proved the validity of the study concept, by demonstrating that some combinations of Xaa and Zaa substitutions in the general sequence R_1-(Ser/Thr)-Xaa-His-Zaa-R_2 provide peptides that are particularly susceptible to specific hydrolysis of the R_1-(Ser/Thr) peptide bond in the presence of Ni(II) ions. We also found that this susceptibility is related to the bulkiness and hydrophobicity of Xaa and Zaa substituents. These results provide a firm basis for subsequent studies aimed at elucidating the molecular mechanism of Ni(II)-dependent hydrolysis and developing this reaction for biotechnological applications.

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