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Dual catalytic role of the metal ion in nickel-assisted peptide bond hydrolysis

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ABSTRACT

In our previous research we demonstrated the sequence specific peptide bond hydrolysis of the R_1-(Ser/Thr)-Xaa-His-Zaa-R_2 in the presence of Ni(II) ions. The molecular mechanism of this reaction includes an N–O acyl shift of the R_1 group from the Ser/Thr amine to the side chain hydroxyl group of this amino acid. The proposed role of the Ni(II) ion is to establish favorable geometry of the reacting groups. In this work we aimed to find out whether the crucial step of this reaction – the formation of the intermediate ester – is reversible. For this purpose we synthesized the test peptide Ac-QAASSHEQA-am, isolated and purified its intermediate ester under acidic conditions, and reacted it, alone, or in the presence of Ni(II) or Cu(II) ions at pH 8.2. We found that in the absence of either metal ion the ester was quickly and quantitatively (irreversibly) rearranged to the original peptide. Such reaction was prevented by either metal ion. Using Cu(II) ions as CD spectroscopic probe we showed that the metal binding structures of the ester and the final amine are practically identical. Molecular calculations of Ni(II) complexes indicated the presence of steric strain in the substrate, distorting the complex structure from planarity, and the absence of steric strain in the reaction products. These results demonstrated the dual catalytic role of the Ni(II) ion in this mechanism. Ni(II) facilitates the acyl shift by setting the peptide geometry, and prevents the reversal of the acyl shift, by stabilizing subsequent reaction products.

1. Introduction

Nickel and its compounds are toxic to humans, *via* inhalatory, dermal and alimentary routes. Contact allergy and respiratory cancers are two major health hazards related to nickel exposure, under environmental and occupational conditions, respectively. While it has become clear that Ni(II) ions are the ultimate toxic species, the exact molecular mechanisms of toxicity are the subject of dispute [1–9].

Nickel(II) dependent peptide bond hydrolysis (in brief, nickel-assisted hydrolysis) is a promising molecular concept for several aspects of nickel toxicity. This sequence specific reaction may impair function of important intracellular proteins, such as zinc finger transcription factors, at the same time yielding stable and reactive Ni(II) complexes, capable of damaging DNA and proteins [10–16]. Peptides susceptible to nickel hydrolysis have a general sequence R_1-(Ser/Thr)-Xaa-His-Zaa-R_2 (where Xaa and Zaa are any amino acids except of Pro and Cys as Xaa, and Cys as Zaa, and R_1 and R_2 are nonspecific N-terminal and C-terminal peptide sequences). The hydrolysis occurs, with an absolute selectivity, at the peptide bond preceding Ser/Thr. Scheme 1 illustrates key steps of the reaction [13,14]. The reaction is enabled by the formation of a square-planar complex in which the Ni(II) ion is bonded by the imidazole nitrogen of the His residue and three preceding amide nitrogens. The first crucial step of the reaction is the N–O acyl shift of the carbonyl group of to hydrolysable R_1-Ser/Thr peptide bond to the Ser/Thr hydroxyl group. This step follows an apparent 1st order kinetic regime (k_1 in Scheme 1). The resulting ester intermediate product (IP) is unstable in water solution, hydrolyzing into two peptides, also according to the apparent 1st order kinetics (k_2 in Scheme 1). The C-terminal peptide product of this reaction step remains bonded to the Ni(II) ion. Therefore, the overall reaction is stoichiometric (1:1), rather than catalytic, with respect to Ni(II). The reaction rate is strongly dependent on the spatial orientations of side chains of amino acids belonging to the Ni(II) coordination site [17], and is strongly (up to a factor of 200) enhanced in the presence of bulky residues C-terminal to the His residue [18]. These facts confirm the notion that crucial role of the Ni(II) ion is largely geometrical. The square-planar coordination mode induces strain on the hydrolysable peptide bond, modulated by sterical crowding of the above mentioned side chains.

In the course of our studies we noted that the stability of IP varies significantly among various peptide sequences studied. In all cases the overall reaction was irreversible, resulting in the full conversion of the substrate into final products. This was assured by the irreversible character of the 2nd step of the reaction, the well-known acid- or base-catalyzed ester hydrolysis [19]. Nevertheless, the data we collected so far did not provide information on the reversibility of the 1st step, the IP formation. This issue can be quite important for many purposes, including the practical application of the reaction for purification of recombinant proteins [20].

We decided to study this issue using a peptide of the sequence Ac-QAASSHEQA-am (Ac- denotes the N-terminal acetylation, and -am the

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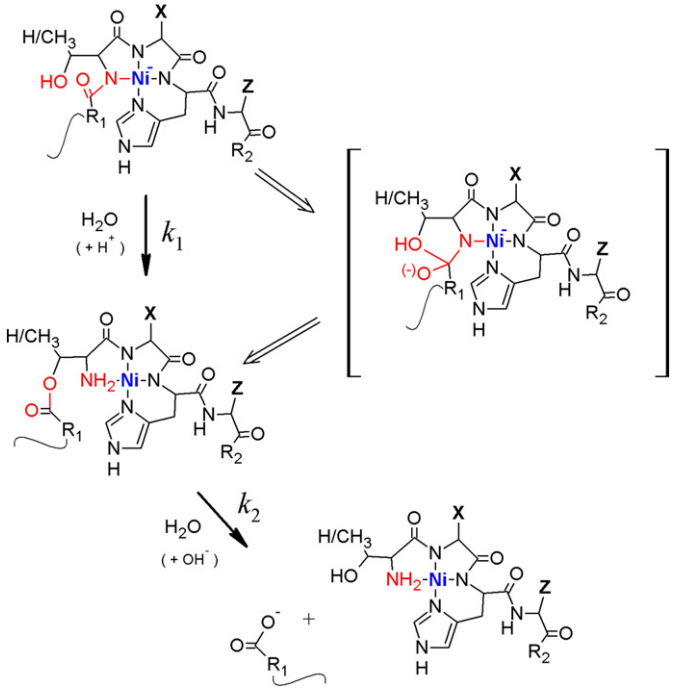

Scheme 1. The molecular mechanism of Ni(II)-assisted peptide bond hydrolysis. The experimentally available rate constants, k_1 and k_2, are associated with respective molecular structures. The postulated tetrahedral transition state is shown in brackets.

C-terminal amidation of the peptide chain), which yields a long-lived and easy to extract IP. This peptide was chosen on the basis of our current research on hydrolysis of filaggrin, a protein involved in the process of outer skin keratinization [21].

2. Experimental

2.1. Reagents

N-α-9-Fluorenylmethyloxycarbonyl (F-moc) amino acids were purchased from Sigma-Aldrich, and Fluka Co. Trifluoroacetic acid (TFA), piperidine, *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU), triisopropylsilane (TIS), N,N-diisopropylethylamine (DIEA) and nickel(II) nitrate hexahydrate were obtained from Sigma-Aldrich. TentaGel® S RAM resin was obtained from Rapp Polymer Inc. Acetonitrile (HPLC grade) was obtained from Rathburn Chemicals Ltd. Pure sodium hydroxide was obtained from Chempur. HEPES (≥99.5%) was purchased from Carl Roth GmbH.

2.2. Peptide synthesis

The Ac-QAASSHEQA-am (substrate, S) and SSHEQA-am (product, P) peptides were synthesized in the solid phase according to the Fmoc protocol [22] using an automatic synthesizer (Protein Technology Prelude). The syntheses were accomplished on a TentaGel S RAM resin, using HBTU as a coupling reagent, in the presence of DIEA. The acetylation of the N-terminus was carried out in 10% acetic anhydride in DCM. In both cases the cleavage was done manually by the cleavage mixture composed of 95% TFA, 2.5% TIS and 2.5% water. S and P were isolated from cleavage mixtures by precipitation by the addition of cold diethyl ether. Following precipitation, peptides were dissolved in water and lyophilized.

S and P were purified by HPLC (Waters) using an analytical C18 column (ACE 250 x 4.6 mm) monitored at 220 and 280 nm. The eluting solvent A was 0.1% (v/v) TFA in water, and solvent B was 0.1% (v/v) TFA in 90% (v/v) acetonitrile. The correctness of molecular masses and purities of peptides were confirmed using a Q-Tof1 ESI-MS spectrometer (Waters). After this step the S and P solutions were frozen in liquid nitrogen and lyophilized.

2.3. UV–vis and CD spectroscopies

UV–visible spectra were recorded in the range of 300–800 nm, on a Cary 50-Bio (Varian) UV–vis spectrometer, using 1 cm cuvettes. The solutions contained 0.95 mM substrate with 0.9 mM Ni(II), 1 mM substrate or a product with 0.9 mM Cu(II), and 0.2 mM intermediate product with 0.18 mM Cu(II), all dissolved in H_2O. The pH of the solution was adjusted manually in the range of 4–12 for Ni(II), and 2–12 for Cu(II), by adding small amounts of concentrated NaOH. The pK_a values for the complex formation were obtained by fitting the absorption value at the band maximum to the Hill equation [23]. Circular dichroism (CD) spectra of Cu(II) complexes with S, IP, and P were recorded in the range of 270–800 nm, on a Jasco J-815 spectropolarimeter, using the same samples as for UV–vis experiments.

2.4. Ni(II) dependent Ac-QAASSHEQA-am peptide hydrolysis

The hydrolysis experiment was performed in a 20 mM HEPES buffer. The concentrations of peptide and nickel(II) nitrate were 0.5 and 2 mM respectively. After setting the pH to 8.2, the samples were incubated at 50 °C in a heating block (J.W. Electronics). These conditions were chosen on the basis of previous studies to optimize the data collection timing [14]. The aliquots were collected at 0, 15, 30, 60, 120, 240, 480 and 1440 min. Control samples, containing peptide and buffer, but without Ni(II), were gathered at the same time points. The 50 μl aliquots were added to 50 μl of 2% (v/v) TFA to stop the hydrolysis reaction (acidification results in the separation of nickel(II) from the Ni(II)-peptide complex). Solutions were stored at 4 °C. For analysis, reaction mixtures were diluted by water 4 to 1 and injected into the HPLC system (Waters), equipped with an analytical C18 column. The eluting solvent A was 0.1% (v/v) TFA in water, and solvent B was 0.1% (v/v) TFA in 90% (v/v) acetonitrile. The gradient conditions are presented in Table 1. The chromatograms were obtained at 220 and 280 nm. Based on the retention times and molecular masses, confirmed using a Q-Tof1 ESI-MS spectrometer (Waters), peaks of the substrate (molecular mass 968.98), the IP (the same mass), and co-eluting final products (molecular masses: 330.34 and 656.65) were identified. The relative amounts of these fractions in each chromatogram were calculated by peak integration using data analysis software Origin 8.1 (OriginLab Corporation).

2.5. Kinetic analysis

To calculate the rate constants k_1 and k_2 for the hydrolysis reaction the set of three equations (Kinet A, Kinet B, and Kinet C) was used, similarly to previous studies [14,17,18].

$$\text{Kinet A} \qquad y = A_0 \times \exp(-k_1 \times x)$$

Table 1
The flow conditions in HPLC separation of reaction substrate, IP and products during Ni(II) dependent hydrolysis of the Ac-QAASSHEQA-am peptide. Solvent A was 0.1% (v/v) TFA in water, and solvent B was 0.1% (v/v) TFA in 90% (v/v) acetonitrile.

Time	% A	% B
0 min	99	1
5 min	99	1
30 min	94	6
31 min	0	100
32 min	0	100
33 min	99	1
40 min	99	1