SYNTHESIS AND ENZYMATIC ACTIVITY OF AN RNase S' ANALOGUE IN WHICH THE 4-IMIDAZOLYLGLYCYL RESIDUE TAKES THE POSITION AND THE ROLE OF HISTIDINE-12*

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1. Introduction

It is generally recognized that histidine-12 plays a direct role in the mechanism of action of ribonuclease A. Thanks to the S-peptide/S-protein system of Richards [1] it has been possible to study the function of the imidazole-12 moiety in the enzymatic reaction by preparing RNase S' analogues in which histidine-12 is replaced by other residues.

Replacement of L-histidine-12 by the isosteric β-pyrazolyl-3-L-alanine [2] or 4-fluoro-L-histidine [3], both having a pK_a^{ring} of 2.5, results in complete loss of catalytic activity, whereas the binding to S-protein remains practically unaffected. These findings demonstrate the influence of the pK_a^{im} on the catalytic efficiency (pK_a^{im} His 6.0). Recently we reported a remarkably high activity of a RNase S' analogue in which His-12 is replaced by L-homohistidine [4,5], that suggestively has almost the same pK_a^{im} value as histidine. The binding capacity of this S-peptide analogue is lowered by a factor of approximately 100 relative to S-peptide 1–20, indicating the influence of lengthening the side chain.

We now wish to report an extension of the investigations on the topochemistry of the active site, consisting of the synthesis of an S-peptide analogue in which the side chain of His-12 is shortened by one carbon atom. The capacity of this peptide to combine with S-protein and ribonucleic acid, and the reactivity of the resulting complex is discussed.

2. Experimental

DL-4-Imidazolylglycine (abbreviated as Nhi) was synthesized according to the procedure described by Schneider [6]. Although L-4-imidazolylglycine can be easily obtained by optical resolution (acylase I) of the DL-compound, the diastereoisomers racemize within several hours under acid as well as alkaline conditions [7]. For that reason it has not much sense to incorporate just one of these isomers into a peptide. One should keep in mind that without a special analysis the ratio of the two products, one with the L- and the other with the D-imidazolylglycine, can only be guessed at.

t-Butyloxycarbonyl-DL-4-imidazolylglycine (Boc-Nhi-OH) was prepared in essentially the same way as described for the synthesis of Boc-histidine [8]; during the reaction with Boc-azide the reaction mixture was kept at pH 10 with 4 N LiOH. Conversion of the di-Boc- into the mono-Boc-derivative was accomplished in MeOH under stirring for 48 hours. After evaporation to dryness H_2O was added and the aqueous solution was brought to pH 3 with 2 N HCl. The acid solution was extracted with *n*-butanol (four times) and the combined extracts were partially evaporated in vacuo to

*Part XIX in the series Studies on Polypeptides; preceding publication on RNase S' analogues: see reference [5].

Abbreviations: RNase A, bovine pancreatic ribonuclease; RNase S, native ribonuclease cleaved by subtilisin; the resulting active complex can be separated into its two inactive components: S-peptide and S-protein. RNase S', the reconstituted active complex obtained by mixing equimolar amounts of S-peptide and S-protein.

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remove H_2O azeotropically, resulting in a spontaneous crystallisation of Boc-Nhi-OH. After filtration crystals were dried (paraffin cuttings) in vacuo at 40°C. Yield 83%; m.p. 186–188 °C. Homogeneous (P^+, RH^+) on TLC (R_f 0.62 in MeOH), when dissolved in a LiOH solution. The nmr-spectrum (DMSO-d_6) was consistent with the structure; δ-values relative to TMS: $(CH_3)_3C$ at δ = 1.4 (s); α-CH at δ = 5.1 (d); π-CH at δ = 7.1 (s); τ-CH at δ = 7.6 (s); NH at δ = 7.0 (d). A satisfactory elemental analysis was obtained ($C_{10}H_{15}N_3O_4$; C,H,N,O).

(Nhi[12])-RNase S-peptide 1–14 was synthesized by the solid-phase method [9] as described previously for the synthesis of other S-peptide analogues [5], starting from 1.5 g of Boc-Asp(OBzl)-resin (0.32 mmol of Boc-Asp (OBzl)-OH per g of Boc-Asp (OBzl)-resin). Boc-Nhi-OH (5eq) was completely coupled within four hours with DCC (5eq) and HOBt (5eq) in DMF. After synthesis and purification a yield of 30 mg was obtained. Homogeneous on paper electrophoresis (pH 4.8 and 6.5) and paper chromatography (R_f 0.70 X His in BAPW 15:3:10:12). An acid hydrolysate showed Lys 1.91(2) Glu 3.26(3), Thr 0.96(1), Ala 2.98(3), Phe 0.91(1), Arg 1.00(1), Nhi 1.07(1), Met 1.03(1), Asp 1.07(1), using Nle as an internal standard; peptide content 70%.

The S-protein activating ability of this S-peptide analogue was determined according to the method of Berger and Levit [10,11]. For further experimental details see reference [5].

3. Results and discussion

Figure 1 shows the activity (change in transmittance/min) of the complex formed when varying amounts of (Nhi[12])-RNase S-peptide 1–14 are mixed with a constant amount of S-protein (5 X 10^{-7} M), using yeast RNA (from BDH, purified by the procedure of Klee and Richards [12]) as a substrate. The S-peptide analogue could not be tested at concentrations higher than 2 X 10^{-4} M under the given assay conditions, due to solubility problems. The highest measured activity was approximately 20% relative to that of RNase S'.

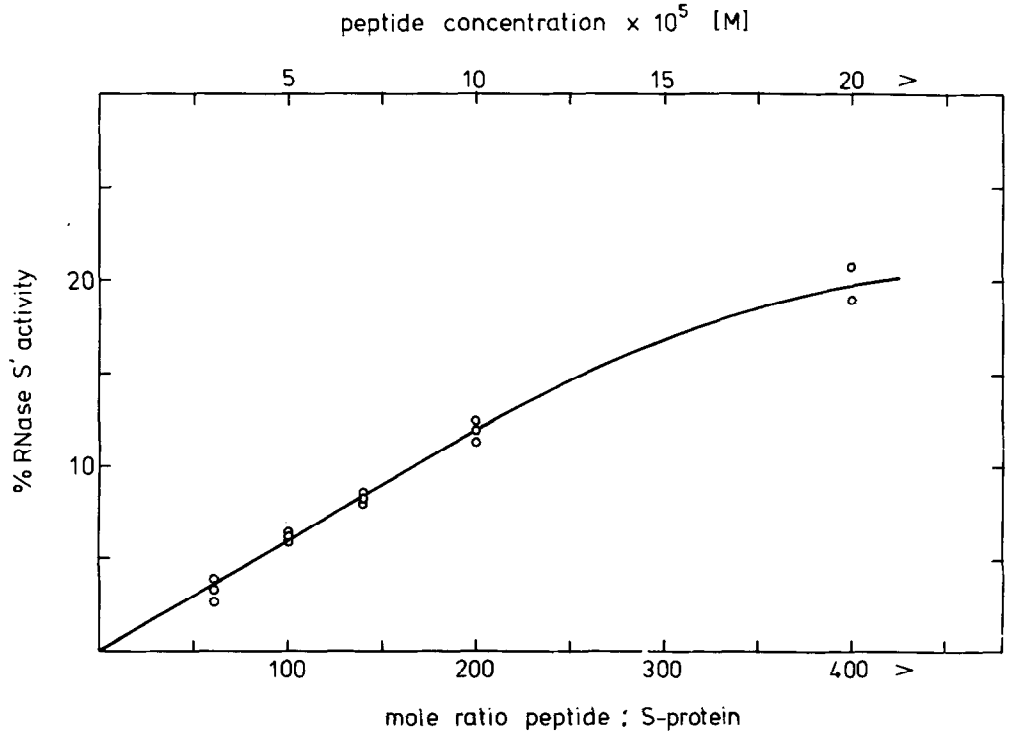

Fig.1. S-protein activating capacity of (Nhi[12])-RNase S-peptide 1–14 relative to RNase S-peptide 1–20 at pH 5.0, 25°C; substrate yeast RNA (0.075% in 0.1 M NaOAc); S-protein concentration 5 X 10^{-7} M.

When (Nhi[12])- and (Hhi[12], Ile[13])-RNase S-peptide 1–14, the last one having a low binding capacity, are mixed with S-protein (5×10^{-8} M) in a molar ratio of 2000:80:1 respectively, no decrease in activity is observed in comparison with the activity measured ($\sim$ 50%) when the Hhi-analogue is combined with S-protein under the same conditions (i.e. close to 50% S-protein saturation). This indicates that neither the L- nor the D-imidazolylglycine[12]-S-peptide acts as an inhibitor. The approximate association constant and the activity of the (Nhi[12])-RNase S′ complex are given in table 1. They were estimated from a double reciprocal plot of the activity measured at a given peptide concentration versus the total concentration of the activating peptide, using the graphical method of Berger and Levit [10,11]. In spite of the relatively low pK_a^{im} of 4.6 [6] and the shortened side chain of the Nhi-12 residue a considerable enzymatic activity can be reached, although the binding capacity of this S-peptide analogue is lowered by a factor of approximately 10^5 in comparison with the natural S-peptide 1–20.

Shortening or lengthening the side chain of His-12 seems to have a strong effect on the association constant of the RNase S′ complex in the presence of yeast RNA as a substrate [13]. Adhering – by way of simplification – to the traditional picture of enzymatic reactions one would describe the results obtained by saying that the S-protein/S-peptide/substrate complex has a somewhat higher free enthalpy in the case of the Nhi- and the Hhi-peptide than with the natural His-12 peptide. Remarkably the rate of the chemical reaction proper is similar in the three cases. Presumably the position and orientation of the reacting nitrogen atom (tele-N?) of the Hhi-12 and the Nhi-12 residue in the complex does not significantly differ from that of the tele-N of His-12 in the normal RNase S′. It is not difficult to envisage that this may go with a lesser fit elsewhere in the complex.

Table 1
Activities (A_{max}) and binding constants (K_b) of some RNase S′ complexes, using yeast RNA as a substrate

Analogue	A_{max} (%)	K_b (M^{-1})
S-peptide 1–20	100	2.5×10^8
S-peptide 1–14	80	5×10^8
(Hhi-12) 1–14	70	2.5×10^6
(Nhi-12) 1–14	80 ± 20	$1.5 (\pm 0.5) \times 10^3$

Evidently the differences in pK_a^{im} of the Nhi, the Hhi and the His residue are not large enough to have a strong effect on the reaction rate. Moreover the differences in basicity will be partly compensated by the differences in degree of dissociation. It seems highly informative to study the pH dependency curves of the three ribonuclease S′ complexes.

For the future one has to reckon with the now disclosed possibility that at least in some enzymes even amino acid residues that directly react in the chemical conversion of the substrate can be appropriately varied without loss of reactivity of the enzyme–substrate complex.

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