ENZYMATIC HYDROLYSIS OF 2',3'-CYCLIC CMP BY HOMOHISTIDINE-12-RIBONUCLEASE S' *

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SUMMARY: Two semisynthetic RNase S' analogues, in which the active site residue histidine-12 is replaced by homohistidine, while in one of them methionine-13 is substituted by isoleucine as well, were studied with respect to their capacity to hydrolyze 2',3'-cyclic CMP, a model substrate for the second step of the RNase catalyzed breakdown of RNA. Kinetic parameters have been determined at pH 6. From the results obtained it is concluded that replacement of histidine-12 by homohistidine has no drastic effect on the catalytic properties of the enzyme in the second step of the reaction.

INTRODUCTION

In previous communications (1,2) we reported on the high enzymatic activity of two RNase S' analogues in which the active site residue histidine-12 is replaced by homohistidine. Using yeast RNA as a substrate, maximum activities of 90% and 80% (relative to S-peptide(1-14)/S-protein) were found on combining synthetic [Hhi^{12}]S-peptide(1-14) and [Hhi^{12},Ile^{13}]S-peptide(1-14), respectively, with RNase S-protein. From these results it was concluded that optimal geometric arrangement for the first reaction step (transphosphorylation) can be realized even when the side chain of His-12 is lengthened by one methylene group. This suggests a conformational flexibility in a region belonging to or situated close to the active site of the enzyme.

To study the enzymatic properties of the [Hhi^{12}] analogues in the second,

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Abbreviations: RNase A, bovine pancreatic ribonuclease (EC 3.1.4.22); RNase S, bovine 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; C>p, 2',3'-cyclic CMP (cytidine 2',3'-cyclic phosphate, sodium salt); Hhi, L-homohistidine [γ-(4-imidazolyl)-L-α-aminobutyric acid].

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hydrolytic, step of the RNase catalyzed breakdown of ribonucleic acids, we determined the kinetic parameters K_m and k_{cat} at pH 6 using C>p as the substrate.

MATERIALS AND METHODS

[Hhi^{12}]- and [Hhi^{12},Ile^{13}]RNase S-peptide(1-14) were synthesized as described earlier (2). C>p was obtained from Aldrich-Europe, Belgium; it contained about 2% impurities (3'-CMP and 2'-CMP) as shown by HPLC. RNase S-peptide and S-protein were purchased from Sigma Chemical Co., U.S.A. and used without further purification. The S-protein and S-peptide(analogue) concentrations were calculated from the weight of compound dissolved. C>p concentrations were determined by measuring the absorbance at 284 nm or 290 nm, depending on the substrate concentration, using molar extinction coefficients of 3680 $M^{-1}cm^{-1}$ and 1260 $M^{-1}cm^{-1}$, respectively. The hydrolytic cleavage of C>p (0.36-6.0 mM in the buffer system 0.05 M Tris, 0.05 M NaOAc and 0.1 M NaCl, I=0.2, pH=6) was followed at 25 °C by the spectrophotometric method of Crook et al.(3) using a Perkin-Elmer EPS-3T spectrophotometer on the 90-110% transmittance scale. The measurements were performed at 284, 290 or 292 nm in quartz cuvettes of 0.2 or 0.5 cm light path, dependent on the substrate concentration. The concentrations of the peptides were chosen high enough to ensure saturation of S-protein (5.2×10^{-7} M) under the assay conditions: S-peptide(1-20), 1.5×10^{-6} M; [Hhi^{12}]S-peptide(1-14), 8.1×10^{-5} M and [Hhi^{12}, Ile^{13}]S-peptide(1-14), 1.7×10^{-4} M. The molar extinction changes for complete enzymatic hydrolysis of C>p were found to be 1290 $M^{-1}cm^{-1}$ at 284 nm, 960 $M^{-1}cm^{-1}$ at 290 nm and 730 $M^{-1}cm^{-1}$ at 292 nm. To determine the velocities at t=0, the progress curves obtained with RNase S', which were found to follow first order kinetics, were plotted semi-logarithmically (3). In the case of the RNase S' analogues the rates of conversion were smaller and the initial velocities could be determined directly from the slope of the (quasi)linear progress curves. K_m and k_{cat} values for the respective complexes were calculated from plots of the initial velocity versus the substrate concentration (see Fig.1), using the non-linear regression procedure of Wilkinson (4).

RESULTS AND DISCUSSION

Values of K_m, k_{cat} and k_{cat}/K_m obtained with the RNase S' complexes are compiled in Table 1. Experimental points and calculated curves (from the data in Table 1) are shown in Fig.1. The [Hhi^{12}]RNase S' complexes exhibit considerable catalytic activity when using C>p, although the relative activities of these analogues are not so high as found with yeast RNA as the substrate. The K_m values are somewhat increased relative to that of native RNase S'. As a result, the values of the catalytic efficiency, k_{cat}/K_m, clearly show the effect of the substitution of histidine-12 by homohistidine.

The measurements were made at only one pH value; thus, the values of the kinetic parameters are apparent ones. Replacement of histidine-12 by homohistidine may shift the bell-shaped pH-profile of k_{cat} and k_{cat}/K_m and the pH de-

Table 1. Kinetic parameters of RNase S' and [Hhi^{12}]RNase S' analogues, obtained from C>p hydrolysis at 25 °C in the buffer system 0.05 M Tris, 0.05 M NaOAc, 0.1 M NaCl, pH=6 and ionic strength 0.2

COMPLEX	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m
RNase S'	1.60 ± 0.04	0.42 ± 0.04	3.80 ± 0.39
[Hhi^{12}]RNase S'	0.39 ± 0.03	3.6 ± 0.5	0.11 ± 0.02
[Hhi^{12},Ile^{13}]RNase S'	0.64 ± 0.05	1.53 ± 0.26	0.42 ± 0.08

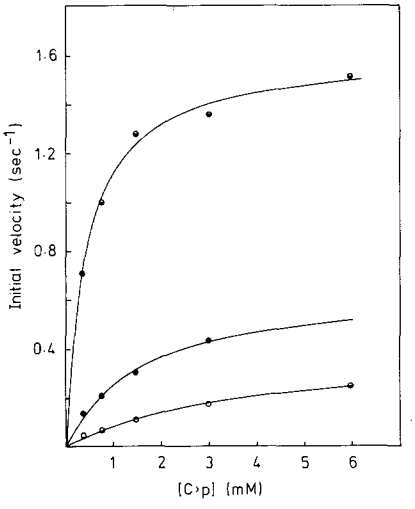

Fig.1. Plots of initial velocity of hydrolysis of C>p versus substrate concentration (pH=6, 25 °C). ⊖ RNase S'; o [Hhi^{12}]RNase S'; • [Hhi^{12},Ile^{13}]RNase S'. Curves calculated from the data in Table 1.

pendence of K_m (5) as a result of changes in the acidity constants of the catalysing acid-base pair, i.e. the side chains of His-12 and His-119. For that reason the [Hhi^{12}] analogues could have more favourable kinetic parameters than indicated in Table 1. Apart from a possible influence of the pH dependence the results obtained with the [Hhi^{12}] analogues may be explained by a

slightly less favourable geometry at the active site during catalysis, resulting in a lower turnover. Additionally, the K_m values indicate that the [Hhi^{12}] RNase S' analogues form a somewhat less stable enzyme-substrate complex with C>p than does native RNase S'. Remarkably, [Hhi^{12},Ile^{13}]S-peptide(1-14) in combination with S-protein appears to be a "better" enzyme (higher k_{cat}, lower K_m) than the corresponding [Hhi^{12}]RNase S' analogue; when RNA is used as the substrate, the reverse is found (2). However, the differences are relatively small. It should be noted that in the case of [Hhi^{12},Ile^{13}]RNase S' a higher peptide concentration is required to completely saturate S-protein, indicating a weaker binding between [Hhi^{12},Ile^{13}]S-peptide(1-14) and S-protein; this finding is consistent with the results of binding studies in the presence of yeast RNA (2).

Summarizing, we conclude that replacement of histidine-12 by homohistidine has a definite but not drastic effect on the catalytic properties of RNase S' in the second step of the degradation of ribonucleic acids, i.e. the hydrolytic opening of the 2',3'-cyclic phosphate to yield the 3'-phosphate.

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